

Interaction of zinc with the yeast
Saccharomyces cerevisiae

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**I certify that this is the true and accurate version of the thesis approved by the
examiners**

Signed.........

Director of Studies

Date.....28/1/06.....

Abstract

Zinc is an essential trace element in biological systems. For example, it activates many enzymes, acts as a cellular membrane stabiliser and is a constituent of the zinc finger proteins that bind specific DNA sequences, playing a critical role in gene expression and genome modification. The present study has focused on the influence of zinc on cell physiology of the yeast *Saccharomyces cerevisiae*. Zinc uptake by industrial strains of *S. cerevisiae*, including brewing strains, and the subsequent utilisation of this key metal during fermentation was studied. Yeast strains take up most of the available zinc very quickly following pitching, with some strains increasing their cellular zinc ten-fold at the onset of fermentation. Zinc content of yeast cell walls was found to remain constant during fermentation and zinc was localised in the vacuole. These and other findings indicated that most of the initial zinc uptake was metabolism-dependent, rather than via a cell surface biosorption phenomenon. After initial periods of cellular zinc accumulation, and during the course of the subsequent fermentation, zinc became virtually undetectable in wort. As yeast cells grew during this period, zinc was distributed to daughter cells at cell division and this effectively lowered their individual cellular zinc concentration. Depending on the extent of yeast growth during fermentation, this may result in the generation of zinc-depleted biomass at the time of yeast harvesting. A brewing yeast strain was also investigated following exposure to stresses typically encountered during the brewing process and a relationship existed between zinc loss from cells and decrease in cell viability during various environmental insults. In order to extend initial laboratory studies to industrial scale, yeast fermentative performance was investigated under various zinc concentrations, firstly in 1 L conical vessels and subsequently in 200 L brewing fermenters. A cell physiological study was also made of zinc-limitation in the haploid reference strain *S. cerevisiae* CEN.PK113-7D in chemostat continuous culture cultivations, in order to pave the way for more in-depth studies aimed at identifying zinc-responsive molecular biomarkers in yeasts. The ultimate aim of such a study was to provide a valuable and rapid way to determine the status of cellular zinc in yeast during fermentation. The consequences of this for efficient industrial processes are discussed including implications for brewing fermentation optimisation based on control of wort zinc bioavailability. Overall, this research has provided new insights into the influence of zinc on yeast cell physiology and the fundamental information gained has practical implications for yeast-based biotechnologies.

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Chapter 1

General Introduction

1.1 *Saccharomyces cerevisiae* and its importance in industrial fermentations

Yeasts have been used for millennia in traditional fermentations of the food industry, such as alcoholic beverages and bread production. In recent years, the capability of yeasts to ferment sugars has also been employed in new biotechnologies such as the production of ethanol as a renewable source of energy (Wheals *et al.*, 1999). Table 1.1 summarises the major characteristics of the fermentation industries employing yeasts. Recent discoveries on the physiology and genetics of these microorganisms have been the starting point for new biotechnological applications. The *Saccharomyces cerevisiae* genome was the first ever from a eukaryote to be mapped (<http://www.yeastgenome.org>). This discovery opened new frontiers to medical research, where yeast cells are used as a model for cytological studies. Advances in molecular biology and genetic engineering, the genetic selection, the capacity to genetically modify yeasts and the relatively easy way to cultivate them, has made these organisms economically attractive to produce drugs, proteins, diagnostics and biologically active products (Russo *et al.*, 1995). One of the latest applications is the employment of recombinant yeasts in immunotherapy for cancer. This technology is based on the capacity yeast cells have to stimulate the immune system to produce highly specific and potent cellular responses against target protein antigens with little toxicity (Franzusoff *et al.*, 2005).

Table 1.1 Summary of major fermentation industry processes employing *S. cerevisiae*

Industry (reference)	Raw materials	Main fermentable carbohydrates	Yeasts employed (all <i>S. cerevisiae</i>)	Fermentation conditions	Inoculum cell number (cells/ml)	Yeast recycling (fermentation system)	Ethanol typical yield (% v/v)
Brewing (Briggs <i>et al.</i> , 2004)	Barley starch malt wort	Maltose Glucose Maltotriose	Ale yeasts Lager yeasts	Ales (18-22 °C) Lagers (8-15 °C)	$\sim 5 \times 10^6 - 2 \times 10^7$	Yes (mainly batch , some continuous)	3-6
Winemaking (Walker, 1999a)	Grapes (must)	Glucose Fructose	Pure selected or spontaneous	Red: 20-30 °C (~7 days) White: 10-18 °C (~7-14 days)	$\sim 10^6 - 10^7$	No (batch)	8-14
Whisky distillery (Watson, 1984)	Barley starch malt wort	Maltose Maltotriose Glucose	Selected commercial yeasts	22°C (start) to 30°C (end)	$\sim 2 \times 10^7$	No (batch)	7-9
Bakery yeast production (Wardrop, 1999)	Sugar cane and beet molasses	Sucrose	Selected commercial yeasts	30°C	$\sim 10^6$	No (fed-batch)	0
Bioethanol (Laluce, 1991)	Sugarcane juice (sucrose), cereals (starch), tuber plants (starch, inulin)	Glucose Fructose Maltose Sucrose	Selected wild yeasts or baker's strains	32-36 °C (6-10 hours)	$\sim 3-5 \times 10^8$	Yes (semi- continuous)	7-11

According to the classification of Kurtzman and Fell (1998), *S. cerevisiae* has been classified in the following taxonomic groups:

Kingdom	→ Fungi
Phylum	→ Ascomycota
Sub-phylum	→ Saccharomycotina (syn. Hemiascomycotina)
Class	→ Saccharomycetes (syn. Hemiascomycetes)
Order	→ Saccharomycetales (syn. Endomycetales)
Family	→ Saccharomycetaceae
Genus	→ <i>Saccharomyces</i>
Type species	→ <i>cerevisiae</i>

Industrial yeasts are usually polyploid strains of *S. cerevisiae*, e.g. beer, wine and baking yeast strains (Pretorius, 2000; Teunissen *et al.*, 2002) or closely related species. Lager brewing strains are allotetraploid, containing two genomes, one from *S. cerevisiae* and one from another *Saccharomyces* yeast, most probably a *Saccharomyces bayanus* isolate. The hybridisation between the two strains occurred in nature spontaneously and the resulting strain has an impaired capacity in meiotic recombination, which results in fertility problems (Kodama *et al.*, 2005). The chromosome structure of lager yeasts has been studied and recently the genome sequence of a specific lager brewing strain, Weihestephana Nr.34, has been completely sequenced (Nakao *et al.*, 2003). Following these discoveries, future studies on gene expression and genomic structural and functional analyses are expected to implement new tools to be used in the brewing industry. For example biomarkers may be used in brewing quality control to determine the physiological status of the cell or to develop new strains capable of better fermentative performance, improved flavour profile and resistance to stresses.

Strains of *S. cerevisiae* have been isolated from natural environments such as vineyards or cavern soils, grape must, *Drosophila* or from anthropic environments such as wineries, breweries or distilleries. Interestingly, however, typical strains of *S. cerevisiae* are rarely if ever present on fruits and berries of plants whereas they widely colonise surfaces of wineries (Phaff *et al.*, 1978; Martini, 1993).

1.2 Sugar metabolism in *S. cerevisiae*

1.2.1 Nutrient uptake

Yeast cells feed, metabolise, grow, reproduce (asexually and sexually), survive and eventually die. The study and understanding of these phases is referred to as yeast cell physiology (Walker, 1998). The physiological responses of yeast cells depend on various factors: the genotype of the yeast strain in question, the physical (e.g. temperature, osmotic pressure) and the chemical conditions (e.g. the bio-availability of nutrients: sugars, amino acids, vitamins and inorganic salts) of its growth environment. Alcoholic fermentation media such as grape must, malt wort and molasses are rich sources of these nutrients.

Nutrient movement within and outwith the cell has to overcome various barriers: the capsular layer (if present), the cell wall, the periplasm, the cell membrane and the intracellular membranes (Walker, 1998). Nutrient molecules of less than 300 Da enter freely through the cell wall whose pores prevent the intake of molecules bigger than 700 Da (Scherrer *et al.*, 1974). Subsequently, nutrients have to face a selective barrier: the plasma membrane whose role is to select compounds, which enter into, or exit from the cell (van der Rest *et al.*, 1995). Four nutrient transport systems are known to work in an active or passive way to allow the movement of the nutrients: free diffusion, facilitated diffusion, diffusion channels and active transport. These mechanisms are schematically described in table 1.2.

The active transport requires the functioning of the plasma membrane H^+ -ATPase. This enzyme expels protons generating a transmembrane electrochemical proton gradient ($\Delta\mu H^+$), negative and alkaline inside, comprising a chemical (ΔpH) and an electrical component ($\Delta\Psi$). This gradient is responsible for the uptake of many nutrients, including ions. The maintenance of a constant transport requires a metabolisable substrate to produce ATP and therefore energy to regenerate the gradient (Jones and Gadd, 1990).

Table 1.2 Transport systems used by yeast cells to translocate nutrients into the cell.

	Type of nutrients	Mechanism	Energy requirements	Speed
Free diffusion	<ul style="list-style-type: none"> - Lipid-soluble solutes - Undissociated organic acids - Short chain alkanes - Long chain fatty acids - Ethanol (Guijarro and Lagunas, 1984) 	Free movement of solutes from high external to low intracellular concentrations (or vice versa, in the case of ethanol which then equilibrates)	No	Very slow
Facilitated diffusion	Some sugars (glucose in <i>S. cerevisiae</i>)	An enzyme specific to the solute (carrier or permease) translocates the solute into the cell	No	Fast
Diffusion channels	<ul style="list-style-type: none"> - Anions, cations (some) - Water (via aquaporins) - Sugar alcohols (glycerol) 	Channels are open when the membrane potential is positive and close when it is negative. Plasma membrane K ⁺ channel is predominant in yeast cells.	No	Fast
Active transport	<ul style="list-style-type: none"> - Majority of nutrients - Some sugars (maltose in <i>S. cerevisiae</i>) 	ATP hydrolyses, due to the enzyme H ⁺ -ATPase, generating an electrochemical gradient. H ⁺ are extruded thanks to the ATP energy. Nutrients may enter together with the protons (symport mechanism) or against the flux of protons (antiport mechanism). Specific permeases can facilitate this process.	Yes	Slow

1.2.2 Sugar transport mechanisms

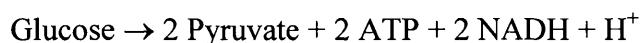
Glucose, fructose, mannose and galactose are hexose monosaccharides which can be utilised by the yeast cells as carbon growth substances. Twenty different hexose transporters (Hxt), part of the facilitator superfamily and with different levels of affinity, are known to transport hexoses across the plasma membrane by facilitated diffusion (Pao *et al.*, 1998). Transcription of the genes encoding for these transporters has been widely studied in chemostat continuous culture under various nutrient-limited conditions by Diderich *et al.* (1999). In a mutant strain lacking these transporters, glucose consumption and transport activity were completely abolished (Wieczorke *et al.*, 1999). Glucose transporters are constitutive in yeast cells and low or high affinity glucose uptake systems (depending on growth respectively in high or low glucose concentrations) may depend on a regulation factor, most probably a kinases (Walsh *et al.*, 1994).

Disaccharides such as sucrose and maltose are also abundant in industrial media. It is generally believed that sucrose fermentation proceeds through extra-cellular hydrolysis of the sugar, mediated by the periplasmic invertase, producing glucose and fructose that are transported into the cells and metabolised. Recent studies on mutant strains of *S. cerevisiae* lacking the major hexose transporters show that these yeasts were still able to ferment sucrose after uptake of this sugar into the cell by an active sucrose transport mechanism (Batista *et al.*, 2004).

Maltose, consisting of two glucose molecules, is the most abundant sugar in barley malt extract and in bread dough. In *S. cerevisiae* the uptake of this disaccharide is due to a mechanism of maltose-one-proton symport (Serrano, 1977). Subsequently maltose is hydrolysed into two molecules of glucose by the enzyme maltase (α -glucosidase). Five genes are responsible for encoding maltose permease and maltase (*MAL1*, *MAL2*, *MAL3*, *MAL4*, and *MAL6*) (Charron *et al.*, 1989). In *S. cerevisiae* glucose levels regulate maltose metabolism, both at the transcriptional and post-transcriptional (enzyme activity) levels (Brondijk *et al.*, 1998; Medintz *et al.*, 2000). High concentrations of glucose repress the high affinity maltose transporter of the Hxt family. Exposure of aerobic, maltose-limited chemostat cultures to excess maltose has even been reported to result in maltose-accelerated death, release of protein and excretion of glucose (Postma *et al.*, 1990). The maltose-accelerated death of *S. cerevisiae* is most likely explained in terms of an uncontrolled uptake of maltose into the cell, resulting in an osmotic shock.

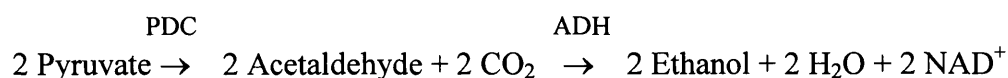
1.2.3 Sugar metabolism: glycolysis, respiration and fermentation

In the yeast cytoplasm, glucose is converted into pyruvate through series of enzymatic reactions called glycolysis (Embden-Meyerhof-Parnas pathway). Other hexose carbohydrates such as galactose, mannose and fructose can also enter into this pathway to be catabolised. The reaction is summarised in the following equation (Walker, 1998):



Energy in the form of ATP and many precursor molecules are generated. In order to maintain the redox state of the cell, NADH must be re-oxidized to NAD^+ . This happens partially during glycolysis where part of dihydroxy acetone phosphate is converted into glycerol.

Without oxygen, NAD must be regenerated by the final stage of fermentation. During this stage, pyruvate is firstly decarboxylated by pyruvate decarboxylase (PDC) and the acetaldehyde formed is finally reduced by alcohol dehydrogenase (ADH) to ethanol or lactic acid (in animals).

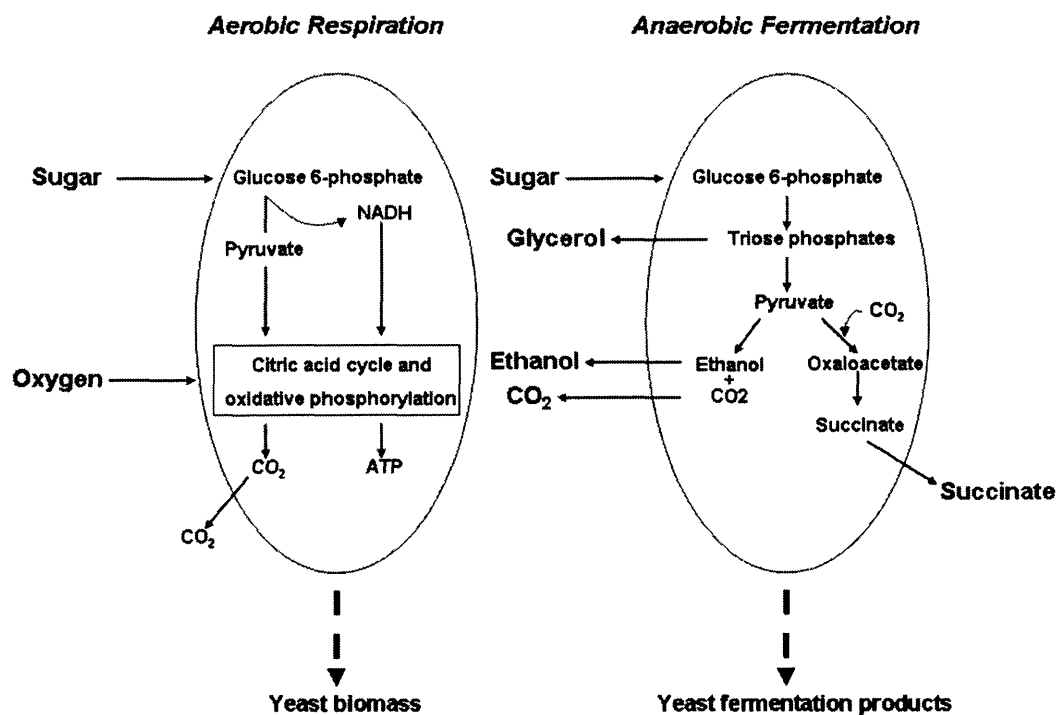


With oxygen, pyruvate enters into the citric acid cycle (TCA) in the form of acetyl-CoA. This cycle is amphibolic because both anabolic and catabolic reactions occur. Many compounds are generated, such as the precursors for the biosynthesis of amino acids and nucleotides and many are subtracted, such as the oxalacetate that is re-established through CO_2 fixation and the glyoxylate cycle. The TCA cycle is summarised by the following reaction:



Normally, during aerobic glycolysis, the electrons of cytoplasmic NADH are transferred to mitochondrial carriers where the oxidative phosphorylation pathway occurs: NADH is re-oxidised to NAD^+ (similarly FADH_2 is re-oxidised to FAD) in the mitochondrial electron transport chain, O_2 is reduced to H_2O and ATP is generated. Part of the unused ATP is dissipated as heat. Fig.1.1 shows the main two pathways yeast cells use to metabolise sugars in the presence or absence of oxygen.

Figure 1.1 Main pathways of sugar utilisation in yeasts (adapted from Walker, 1998).



1.2.4 Control of sugar metabolism: Pasteur and Crabtree effect

Oxygen is a key environmental factor that regulates sugar metabolism in yeast cells. In *S. cerevisiae* two main phenomena are known to regulate the sugar metabolism in presence of oxygen: the Pasteur effect and the Crabtree effect whose main characteristics are summarised in Table 1.3. The ability of the yeast *S. cerevisiae* to readily degrade sugars to ethanol and carbon dioxide, even under aerobic conditions when the glucose concentration in the media exceeds 0.8 mM (Verduyn *et al.*, 1984), is the basis of the Crabtree effect. In contrast, at low external glucose concentrations and under aerobic conditions, for instance, during growth in glucose-limited chemostat cultures, ethanol is not produced and carbon dioxide is the main catabolic carbon product (Postma *et al.*, 1989b). Under these conditions, glucose is catabolised slower than in anaerobic conditions and this is the basis of the Pasteur effect. For certain industrial applications, such as baker's yeast production, it is important to control the carbon dioxide levels and the ethanol produced in aerobic conditions. This explains why molasses medium is delivered to the growing cells in controlled fed-batch propagators to maximise respiratory growth and avoid any ethanol production.

Table 1.3 Main phenomena controlling sugar metabolism in *S. cerevisiae*

Phenomenon	Description	Cell status	Culture conditions	Possible explanation
Pasteur effect	Inhibition of sugar consumption by aerobiosis	Resting or nutrient starved cells	Glucose level below 0.8 mM	Higher affinity for pyruvate, by pyruvate dehydrogenase (respiratory pathway) than by pyruvate decarboxylase (fermentative pathway) (Holzer, 1961).
Crabtree effect	Fermentation predominates over respiration in aerobic conditions	Cells in steady state (long term Crabtree)	Aerobic sugar-limited cultures (glucose 1.5%) at high specific growth rates $D=0.3\text{h}^{-1}$ (long term)	Limited respiratory capacity resulting in overflow metabolism at pyruvate level. (Postma <i>et al.</i> , 1989a). Glucose repression of respiration genes.
		Cells in steady state (short term Crabtree)	Aerobic sugar-limited cultures (glucose 0.5%) at $D=0.1\text{h}^{-1}$. Sudden excess level of glucose added to the medium (short term)	High pyruvate decarboxylase and ADH activity, increased glucose uptake from the environment (van Urk <i>et al.</i> , 1990). Glucose inactivation of respiration enzymes.

1.3 Metal ions and yeast physiology

1.3.1 The role of metal ions in yeast cells

Yeasts require mineral nutrients at precise concentration for cellular growth and metabolism (Jones and Greenfield, 1984).

Bulk metals, such as potassium and magnesium, are required at millimolar concentrations (milligrams/litre), whereas trace elements, such as calcium, zinc, manganese, iron and copper are required at micromolar concentrations (micrograms/litre). Other ions could be toxic at very low concentrations (micrograms/litre): Pb, Cu, Cd, Cr, Hg, Ni, and Al. The utilisation of such ions by yeast cells is often influenced by many factors including metal ion deficiency or excess, chelating/absorbing material in the media and the presence of other ions acting as antagonists (Jones and Gadd, 1990).

Metals play structural roles in cellular organelles, proteins and phospholipids and influence cell-cell interactions in the phenomenon of flocculation. Metals are also required in nutrient uptake (e.g. Mg-ATPase), gene expression, cell division, growth, fermentation and in energy maintenance. At defined concentrations, some metals may help cells to cope with environmental stresses (Walker, 2004). Table 1.4 summarises the principle functions of essential ions in yeast physiology.

Regarding fermentation, for example, it has been hypothesized that intracellular magnesium may stimulate the activity of pyruvate decarboxylase in brewing strains of *S. cerevisiae* (Smith, 2001) while zinc is well known as an essential ion for alcohol dehydrogenase (ADH) (Magonet *et al.*, 1992), which facilitates the conversion of acetaldehyde into ethanol at the end of fermentation.

If present at high concentration, calcium ions may be detrimental and inhibit both growth and fermentation, due to the antagonism of calcium with magnesium (Walker *et al.*, 1996). Although yeast cells actively extrude calcium, this metal plays a key role in the phenomenon of flocculation by stabilising bridges between lectin proteins of the cell wall and carbohydrates receptors on another cell, thus facilitating adhesion between adjacent cells (Miki *et al.*, 1982). In brewing, such a capacity to form flocs is used to facilitate the separation between cells from immature (green) beer at the end of fermentation. In top fermenting yeasts, in traditional ale production, flocs rise to the surface and are removed through skimming or suction. In bottom fermenting yeasts, in lager production, flocs precipitate at the bottom to be subsequently removed. Cold temperatures promote this flocculation phenomenon (Briggs *et al.*, 2004).

With regard to chemical and physical stresses encountered during industrial fermentation processes, magnesium protects cells from osmostress (D'Amore *et al.*, 1988b), ethanol (Birch and Walker, 2000), toxic metals (Blackwell *et al.*, 1997; Karamushka and Gadd, 1994) and the oxidant effect of free radicals (Szantay, 1995). As a consequence, Mg-enriched cells retain viability and vitality under stress. Although an antioxidant role has been ascribed to zinc (Truong-Tran *et al.*, 2001), to date, no information has been provided on how this metal may protect yeast cells from industrial stresses.

Table 1.4 Functions of essential ions in yeast physiology (adapted from Walker, 2004)

Metal ion	Optimal concentration in growth medium*	Main cellular function
Macroelements:		
K	2-4 mM	Osmoregulation, enzyme activity
Mg	2-4 mM	Enzyme activity, cell division
Microelements:		
Ca	<μM	Second messenger, yeast flocculation
Fe	1-3 μM	Haem—proteins, cytochromes
Zn	4-8 μM	Enzyme activity, protein structure
Mn	2-4 μM	Enzyme cofactor
Cu	1.5 μM	Redox pigments

*Values depending on strain, culture conditions and presence of chelators in the media.

1.3.2 Transport of metal ions in yeast

Yeast intracellular ionic composition is different from that of the external medium. As a consequence, yeast cells have developed a system of transporters in order to face environmental changes and survive when nutrients are limited or when they are in excess.

Metal ion uptake into the cell occurs in two stages: biosorption and absorption/translocation. The former is also referred to as metabolic-independent phase while the latter as metabolic-dependent phase (Table 1.5).

Table 1.5 Two stage uptake system in yeast cells (Mowll and Gadd, 1983)

Phenomenon	Description	Energy requirement	Cell localisation
Biosorption	Unspecific binding of the ions to the cell wall. Cells may be dead.	No	Cell wall
Absorption	Transport is aided by a proton-pumping ATPase	Yes	Plasma membrane (then translocation to cytosol)

The metabolic status of cells and the prevailing growth conditions are essential to determine the cellular capacity of yeasts to uptake metals. Other factors such as low temperature, metabolic inhibitors and absence of energy-yielding substrates are known to negatively influence uptake. After an initial phase of passive cell wall binding, the transport of divalent cations is dependent on plasma membrane H^+ -ATPase (Jones and Gadd, 1990). The membrane potential also influences metal ion transport. For example the cell may extrude K^+ to increase polarisation of the plasma membrane in order to facilitate ion uptake. This has been documented in lager yeast *S. carlsbergensis*, where the uptake of Mn^{2+} , Mg^{2+} and Zn^{2+} was accompanied by efflux of K^+ (Okorokov *et al.*, 1983; Jones and Gadd, 1990). High extra-cellular concentrations of divalent cations may also inhibit the uptake when the levels are close to the saturation.

The transport of an ion across a membrane is achieved thanks to specific sets of transporters. Various metal ion transport systems are known and sometimes, for the same ion, more uptake systems with different affinities have been described, as will be explained below for zinc (section 1.5.3). Interestingly, for iron, *S. cerevisiae* has developed two mechanisms of uptake. One of them is known as an “opportunistic strategy of iron uptake” using siderophores, low-molecular-mass compounds with a high affinity for ferric ion, which are secreted in response to low-iron environments. *S. cerevisiae* is not able to synthesise them but has developed an energy dependent transport system for the uptake of siderophores produced by other microorganisms (Lesuisse *et al.*, 2001).

The main genes responsible for encoding such transporters have been recently identified. This area of research is relatively new and since *S. cerevisiae* genome was completely mapped in 1996, this organism has become the main eukaryotic model for the understanding of human diseases related to abnormal ion uptake (Nelson, 1999). The encoding genes for the main ion transporters are presented in Table 1.6

Table 1.6 Main genes encoding transporters for metal uptake in yeast cells

Element	Genes	Comments
Zn	<i>ZRT1/2, ZRT3</i>	High and low affinity system. Also vacuolar transport
Fe	<i>FRE1/2/3/4/5/6/7</i> <i>FET3, FTR1</i>	Fet4p is a metal transport also for Zn^{2+} , Mn^{2+} and Cu^{2+}
Mn	<i>SMF1/2/3</i>	Also for iron. Only high affinity system
Cu	<i>CTR1, 2, 3</i>	High and low affinity system
Mg	<i>ALR1/2, MRS2</i>	Membrane and mitochondrial transporters
Ca	<i>PMR1, PMCI</i>	Golgi and vacuole Ca^{2+} -ATPase

Information from Van Ho *et al.*, 2002; Walker, 2004.

1.3.3 Metal ion homeostasis in yeast

Once the ions have crossed the plasma membrane, many mechanisms work together to ensure that cellular ion homeostasis is maintained, through highly regulated processes of uptake, storage and secretion (Nelson, 1999). The understanding of these mechanisms is fundamental for industrial media optimisation required for processes aimed at maximising biomass (e.g. dried yeast starter cultures for winemaking) and for production of fermentation compounds (ethanol and numerous secondary flavour congeners).

More precisely, the object of such mechanisms is two-fold: to prevent accumulation of the metals in the freely reactive form (metal detoxification pathways) and to ensure proper delivery of the ion to target metalloproteins (metal utilisation pathways).

Some ions may be sequestered in the nucleus, or in the cytoplasm through polyphosphates or calmodulin which is a protein serving as the major intracellular receptor for calcium and which mediates many effects of this ion (Cyert, 2001). Metallothioneins, cysteine-rich proteins of low molecular weight, are also known to bind various heavy metals minimising their toxicity. Metallochaperones are soluble proteins that ensure the safe transfer of ions (e.g. copper and probably iron) to cytoplasmic sites where they are required (O'Halloran and Culotta, 2000). Some ions may be compartmentalised in organelles for later utilisation, e.g. in the mitochondrion, or in the Golgi apparatus, as for manganese which is delivered to sugar transferases (Culotta *et al.*, 2005). Other ions, such as zinc, magnesium and manganese can be

stored or detoxified in the vacuole. In the case of zinc, this will be dealt with in detail in section 1.5.3. In some cases ions may remain free in the cytoplasm at very low concentrations to become available for metabolic functions. For example in yeast free copper can be kept at the concentration of one atom per cell (Rae *et al.*, 1999).

In industry, the capacity to accumulate and store metals in the vacuole or other compartments can be used to pre-condition yeast cells with metals prior to pitching. This may be a good approach to solve the problem of adding metals directly to the industrial medium. Such practises may be prohibited by law as in the case of the German purity law (Reinheitsgebot) adopted in 1516 and stating that the only ingredients used for the brewing of beer must be barley, hops, water and yeasts. The latter was not originally included in the first version of the law since at that time, the role of yeasts in fermentation was not then known. Capacity to accumulate high cellular concentrations of metals such as magnesium and zinc has been employed in commercial preparations based on non-viable metal enriched yeast cells that can be supplemented to the medium during fermentation as a source of ion. For example in brewing, one of the products available is “*Servomyces*” from Lallemand Inc. *Servomyces* is a dried single-strain brewing yeast, available as dead or viable cells, pre-enriched with zinc during the propagation process, whose zinc concentration reaches 50,000-55,000 ppm. Once supplemented to the fermentation medium, the product improves zinc bio-availability, decreases fermentation time, increases yeast sedimentation and reduces harsh sulphur notes. Therefore, resulting beer is claimed to be smoother and more balanced (<http://www.lallemand.com>).

1.4 Zinc and its importance in biology

1.4.1 Zinc chemistry and methods for zinc determination

Zinc is a transition and group II element with atomic number 30 and an atomic weight of 65.37 Daltons. Under physiological conditions zinc is very stable and exists in the divalent state. It is not redox active, since neither the potential oxidised form Zn^{3+} , nor the potential reduced form Zn^+ occur in cells. Zinc is able to form bonds with many molecules including sulphur from cysteine, nitrogen from histidine and oxygen from glutamate, aspartate and water (Berg and Shi, 1996). The flexibility of its coordination sphere allows zinc to form a variety of complex geometries and to participate in enzymatic oxido-reduction reactions in coordination with organic cofactors (Vallee and Aulde, 1992).

Various techniques for metal analyses are available, each having its own particular advantages and disadvantages. Detection limits, sensitivity and sample size are key factors to the choice of a technique. AAS (Atomic Absorption Spectroscopy) is the most widely used technique for trace element analyses. It is based on the principle that heating vaporises the sample into free atoms. A certain wavelength of light, characteristic for each element, is passed through the vapour. As the amount of ground state atoms in the path increases, the amount of light absorbed also increases so that measurements of light absorbed permits a quantitative determination of the amount of analyte present (Sanui and Rubin, 1982). Since the wavelength absorbed is specific for each element, no interference occurs with other elements. Matrix interferences may arise if the sample detected is rich in dissolved solutes, such as carbohydrates or phosphates. The latter are especially problematic for calcium analyses (Hughes and Poole, 1989).

Zinc and other elements as Ca, Mg, Co, Ni, Mn may also be investigated by the use of a flameless atomic absorption technique. The flame is replaced by a small electrically-heated graphite tube. This technique has been suggested for the determination of metals in brewing materials, being of high sensitivity and simplifying sample preparation (Helin and Slaughter, 1977a). More recently an international group of researchers is evaluating the possibility of using the inductively-coupled plasma emission spectroscopy as an alternative method to AAS for the analyses of zinc and other elements in wort and beer (Sedin *et al.*, 2004).

Table 1.7 summarises the principal techniques available for metal ion detection.

Table 1.7 Principal techniques employed in metal ion analyses (from Hughes and Poole, 1989).

Technique	Principle	Advantages	Problems	Detection limit (e.g. Zn, ppb)
Atomic Absorption Spectroscopy (flame AAS)	Measurements of the intensity of the absorbance of the light beam	No interferences from other elements	<ul style="list-style-type: none"> • Interference from chemical material in the samples. • Volume sample of several ml 	10
Atomic Absorption Spectroscopy (graphite furnace) AAS)	As above but heating is generated by a graphite furnace.	<ul style="list-style-type: none"> • Few hundred μl required • Very low detection limits 	Introduction of sample may be the main source of imprecision	0.001
Atomic Emission Spectroscopy (AES)	Atoms are excited to a higher electronic state. The emission of radiation due to the subsequent fall to the ground state is related to defined range of concentrations. Heat generated by flame or plasma discharges	<ul style="list-style-type: none"> • 80% of the elements can be analysed • No interferences • Simultaneous analyses of a range of elements 	High volume of sample required compared to graphite furnace AAS	1
Inductively coupled plasma source mass spectrometry (ICPSMS)	Elemental abundances or isotopic ratios are determined by the mass spectrometry (MS) of ions generated in an inductively coupled Ar plasma (ICP). Analyte isotopes are separated according to their mass/charge ratio by a mass spectrometer and detected and measured by a detector (an electron multiplier).	<ul style="list-style-type: none"> • High ionisation efficiency • High sensitivity • High speed • No interferences 	Ions can be double ionised	0.1
Ion chromatography (IC)	Metals are separated using ion exchange column and eluent containing a complexing agent. Detection by conductivity or optical methods	Analyses of metals in different oxidation state and with different coordination environment	<ul style="list-style-type: none"> • Choice of the correct columns and eluent 	10
Stripping voltammetry (SV)	Reduction of the metallic species at a controlled potential using a hanging drop mercury electrode is followed by the electrochemical oxidation or stripping of the reduced metal	<ul style="list-style-type: none"> • Low detection limits • No interference problem 	<ul style="list-style-type: none"> • Preparation of the working electrode • Possible shift or distortion of peaks due to formation of intermetallic compounds, e.g. Zn, Cu (Wise <i>et al.</i>, 1983) 	<1

New techniques, based on the application of fluorescent probes (fluorophores), are available for the analyses of free zinc in biological systems that allow the combination of chemical with spatial information obtained observing the cells microscopically (Thompson, 2005). After the use of the first fluorescent dye TSQ (Frederickson *et al.*, 1987) and the publication on zinc in biological systems of Berg and Shi (1996) regarding the key role of this metal in zinc finger domains, the interest for zinc in general and the techniques to visualise it have become widespread. Consequently, new fluorophores have been developed in the last ten years (see Table 1.8). They have been used for zinc detection in neuron studies (Sensi *et al.*, 1997) and more recently to determine zinc localisation in *S. cerevisiae* (MacDiarmid, 2003; Devirgiliis, 2004) and Mg localisation in *Schizosaccharomyces pombe* (Zhang *et al.*, 1997)

Cellular free zinc may vary depending on the type (neurons, blood cells, yeasts, etc.) and status of the cell. Its cellular concentration usually ranges from femtomolar to millimolar but this latter level may occasionally drop in the range of picomolar to nanomolar concentrations (Outten and O'Halloran, 2001). Such a range of small concentrations and the interference with other ions present at micromolar level has made the research of new fluorophores particularly difficult: selectivity, photostability, wider range of excitation and emission wavelengths and the capacity to enter easily into the cells are the most desired, researched characteristics.

Many of these probes are now commercially available, such as Newport green DCF, Fluo-Zinc 3 and Rhod-Zin 1, from Molecular Probes Invitrogen (<http://probes.invitrogen.com>). These fluorophores are available in the cell-permeable form and are easily absorbed by the cells. The molecule is actively de-esterified and the active-chelating part subsequently forms specific bonds with zinc (Tsien, 1981). Once excited at a precise wavelength, the chelated molecule passes from a ground to an excited state, which is kept for a short time, called state lifetime. Eventually the probe returns to the original ground state emitting a photon of energy (fluorescent emission).

Table 1.8 shows the main chemical and optical properties of the probes now available for zinc from Molecular Probes. K_d is the parameter measuring the affinity to a specific ion: higher K_d , the lower is the affinity (as for Ca^{2+} , the element that interferes mostly with Zn^{2+}).

Table 1.8 Optical and physical properties of the main probes available for zinc analyses (adapted from Gee *et al.*, 2002 and Probes Invitrogen website handbook <http://probes.invitrogen.com/handbook/>).

<i>Probe</i>	<i>Ex (nm)</i>	<i>Em (nm)</i>	<i>K_d (Zn²⁺)</i>	<i>K_d (Ca²⁺)</i>
Rhod Zin-1	555	575	23 μ M	35mM
Rhod Zin-3	550	575	65 nM	35mM
Newport green DCF	505	535	1 μ M	>100 μ M
Fluo-Zin-3	494	516	15 nM	b
Fura Zin-1	325	375/510 ^a	3 μ M	40 mM
Indo Zin-1	345/405 ^a	475	3 μ M	65 mM

^a the first number represents the fluorescence excitation maximum, while the second number represents the fluorescence emission maximum

^b No fluorescence response to <10 mM Ca²⁺ was observed.

1.4.2 The biological roles of zinc

In biological systems zinc is an essential trace element, estimated to be required for almost 3% of the yeast proteome function (Eide, 1998). Its abundance as a transition metal is second only to that of iron (Vallee, 1988). It is now known to be an integral component of a large variety of proteins and enzymes, being indispensable for the function of nearly 300 of them (Vallee and Auld, 1990) (see Table 1.9): e.g. acid and alkaline phosphatase, aldolases, Cu,Zn-superoxide dismutase, 3-phosphate dehydrogenase and alcohol dehydrogenase (Leskovac *et al.*, 2002). However, zinc plays a purely structural role only in few enzymes (Berg and Shi, 1996). Zinc participates in a wide variety of metabolic processes including carbohydrate, lipid, protein and nucleic acid synthesis or degradation. Zinc ions can form bridge bindings between lipid molecules reducing the capacity of the phosphate groups bound to zinc to take up water. These bindings make the membrane surface more hydrophobic and rigid (Binder *et al.*, 2000), thus influencing membrane fluidity (Garcia *et al.*, 2005). Excess zinc can interfere with respiration at the level of mitochondrial aconitase activity (Rhodes and Klug, 1993; Costello *et al.*, 1997). It is the main constituent of the zinc finger proteins that bind specific DNA sequences, playing a critical role in gene expression and genome modification (Rebar and Miller, 2004). This property is mainly due to the lack of redox activity of zinc which stabilises DNA and RNA molecules and

avoids any radical reaction resulting in nucleic acid damage (Berg and Shi, 1996). Zinc is also critically placed to control apoptosis, suppressing major pathways leading to this phenomenon. Apoptosis is an active energy dependent process of programmed cell death, closely linked with oxidative stress. Zinc acts as a cytoprotectant and minimises oxidative damage by stabilising both lipids and proteins, increasing glutathione, the main cellular anti-oxidant, and by suppressing the activation of caspase-3, protease that cleaves to substrates causing critical morphological changes (Truong-Tran *et al.*, 2001). Zinc toxicity in plant cells can determine high chromatin material condensation, loss of cytoplasm structure, disintegration of cell organelles and of the vacuole development. High zinc level results in an increase of the number of nucleoli and in the synthesis of proteins involved in heavy metal tolerance (Rout and Das, 2003).

Zinc is fundamental in human nutrition. Since the body of an adult human contains 2-3 grams of zinc, this metal is one of the most prevalent “trace” elements (Berg and Shi, 1996). Zinc dietary deficiency is linked to hypogonadism, oxidative damage, alterations in the immune system, hypogeusia, neuropsychological impairment, dermatitis (Mafra and Cozzolino, 2004), Alzheimer’s disease, epilepsy and ischemic stroke (Bush *et al.*, 1994; Koh *et al.*, 1996). Zinc administration to human subjects results in decrease of lipid peroxidation products and DNA adducts (Prasad *et al.*, 2004). Chronic zinc deprivation generally results in increased sensitivity to some oxidative stress (Powell, 2000). Usually, zinc deficiency in nutrition is not common in industrial countries but alcoholism, malabsorption, sickle cell anemia, chronic renal disease and chronically debilitating diseases are known to be predisposing factors (Prasad, 1984).

Zinc deficiency is the most common micronutrient deficiency problem in cereals. Worldwide research has shown that 49% of 190 soils from 25 countries were deficient in zinc, regardless of cold or warm climates, drained or flooded soils and acid or alkaline soils (Ruel and Bouis, 1998). Zinc depletion in plants causes symptoms at the morphological level such as leaf mottling, small leaves, and elongation repression of leaves and/or stems as well as at physiological level by decreasing protein content and accumulation of free amino acids and amides (Obata *et al.*, 1996). Several ways have been proposed to overcome zinc deficiency: increasing the concentration of zinc in soils, reducing the amount of phytic acid (inhibitor of zinc absorption) and raising the concentration of sulphur-containing amino acids which promote zinc absorption (Ruel and Bouis, 1998). However, some plants are known to grow very well in zinc deficient

soils exhibiting very good zinc efficiency (ZE). The ability to maintain the activity of zinc requiring enzymes in response to zinc deficiency may be a possible explanation for such high ZE (Hacisalihoglu and Kochian, 2003).

Free zinc levels in bacteria are maintained at the femtomolar concentrations under steady state conditions (Outten and O'Halloran, 2001). Consequently, bacteria have developed regulatory mechanisms to maintain zinc homeostasis. In *Bacillus subtilis*, three zinc (II) uptake systems have been identified: *ycdHI-yceA*, *yciABC* and *zosA* (Moore and Helmann, 2005). When zinc levels become toxic, sequestration, active efflux and in some cases both mechanisms can co-exist (Choudhury and Srivastava, 2001).

Table 1.9 List of enzymes in microbes, whose function is controlled by zinc (adapted from Valee, 1988). A: catalytic role, B: structural role, C: regulatory role, ?: unknown, *letters in parentheses*: roles fulfilled by metals other than zinc.

Name	Source	Role
Class I: Oxidoreductases		
Alcohol dehydrogenase	Yeast	A
D-Lactate dehydrogenase	Bacteria	?
D-Lactate dehydrogenase	Yeast	?
Superoxide dismutase	Fungi, bacteria	A
Class II: Transferases		
Transcarboxylase	<i>Propionibacterium shermanii</i>	?
Aspartate transcarbamylase	<i>Escherichia coli</i>	B
Phospholuciferase	Yeast	?
RNA polymerase	Yeast, protests, bacteria	A
Mercaptopyruvate sulfur transferase	<i>E. coli</i>	?
Class III: Hydrolases		
Alkaline phosphatase	Bacteria	A(C)
5' Nucleotidase	Bacteria	?
Phospholipase C	<i>Bacillus cereus</i>	A
Cyclic nucleotide phosphodiesterase	Yeast	?
Nuclease	Microbes	?
α -amylase	<i>Bacillus subtilis</i>	B
Aminopeptidase	Fungi, bacteria	AC
Dipeptidase	Bacteria	A
DD-Carboxypeptidase	<i>Staphylococcus albus</i>	A
Elastase	<i>Pseudomonas aeruginosa</i>	?
Neutral protease	Fungi, bacteria	A(B)
Collagenase	Bacteria	A
Aminocyclase	Microbes	?
β -Lactamase II	<i>B. cereus</i>	A
Inorganic pyrophosphatase	Yeast	?
Nucleotide pyrophosphatase	Yeast	A
Class IV: Lyases		
Fuctose-1,6-bisphosphate aldolase	Yeast, bacteria	A
L-Rhamnulose-1-phosphate aldolase	<i>E. coli</i>	A
Glyoxalase I	Yeast	A
Class V: Isomerases		
Phosphomannose isomerase	Yeast	?
Class VI: Ligases		
tRNA synthetase	<i>E. coli</i> , <i>B. stearothermophilus</i>	A
Pyruvate carboxylase	Yeast, bacteria	?

1.5 The role of zinc in yeast cell physiology

1.5.1 Zinc effects on yeast growth and metabolism

Zinc plays a central role in the enzyme function in yeast cells. Special regard should be made to alcohol dehydrogenase (ADH) stimulated by this metal. In yeast cells, zinc increases riboflavin biosynthesis and stimulates the uptake of some sugars such as maltose and maltotriose, at very high concentrations (300 ppm) and only in some brewing strains (Rees and Stewart, 1998). Zinc is also required for endoplasmic reticulum function in *S. cerevisiae* (Ellis *et al.*, 2004). The average zinc cellular content in yeast cells has been defined to be 0.12 g/kg dry weight (Jones and Greenfield, 1984), this value being strain and growth condition dependent.

Zinc deficiency depresses yeast growth with cells tending to swell and to form clusters (Obata *et al.*, 1996). Zinc depletion in *S. cerevisiae* also results in a decrease of the activity of phospholipid synthesis and subsequent alteration of phospholipid composition (Iwanyshyn *et al.*, 2004).

At high concentration, zinc may become toxic. Zinc toxicity has effects on the inhibition of H⁺ efflux and K⁺ uptake, reduction of viability, K⁺ efflux and changes in the kinetics of Zn²⁺ uptake (White and Gadd, 1987). Rees and Stewart (1998) have explained such toxicity with various hypotheses: formation of binding with inappropriate sites in proteins or cofactors, activation of degradative enzymes, denaturation of enzymes, induction of autolysis by activating proteolysis, inhibition of transport systems for essential ions and nutrients and changes in the fluidity of the plasma membrane.

1.5.2 Zinc transport systems in yeast

Zinc is a hydrophilic, highly charged ion, which cannot cross biological membranes by passive diffusion, requiring to be actively transported (Guerinot and Eide, 1999). In *S. cerevisiae*, zinc uptake and homeostasis are controlled both at transcriptional and post-translational levels. The transcriptional system operates at moderate intracellular zinc levels, between 0.01 and 0.07 nmol of Zn/million of cells, and the post-translational operates when intracellular zinc levels are above 0.07 nmol of Zn/million of cells (Zhao *et al.*, 1998; Gitan *et al.*, 1998).

At the transcriptional level, three or more uptake systems are known to control zinc uptake. One system has a high affinity (K_d = 10 nmol/L) for zinc and is active in zinc limited cells (Zhao and Eide, 1996a). A second system has a lower affinity (K_d = 100

nmol/L) for zinc, and is active in zinc-replete cells (Zhao and Eide, 1996b). These systems are very specific for zinc and are not involved in uptake of other metals.

One transport protein works for each of the two systems: Zrt1 for the high affinity system and Zrt2 for the low affinity system. These proteins are localised in the plasma membrane and share 44% of identity in the amino acid sequence and 67% of similarity (Eide, 2003). A third system is also known to take part to zinc uptake: the transport protein Fet4. Fet4 is not only zinc specific but is also involved in iron and copper uptake (Waters and Eide, 2002). This protein, together with Zrt1 and Zrt2, belongs to the ZIP family of metal ion transporters.

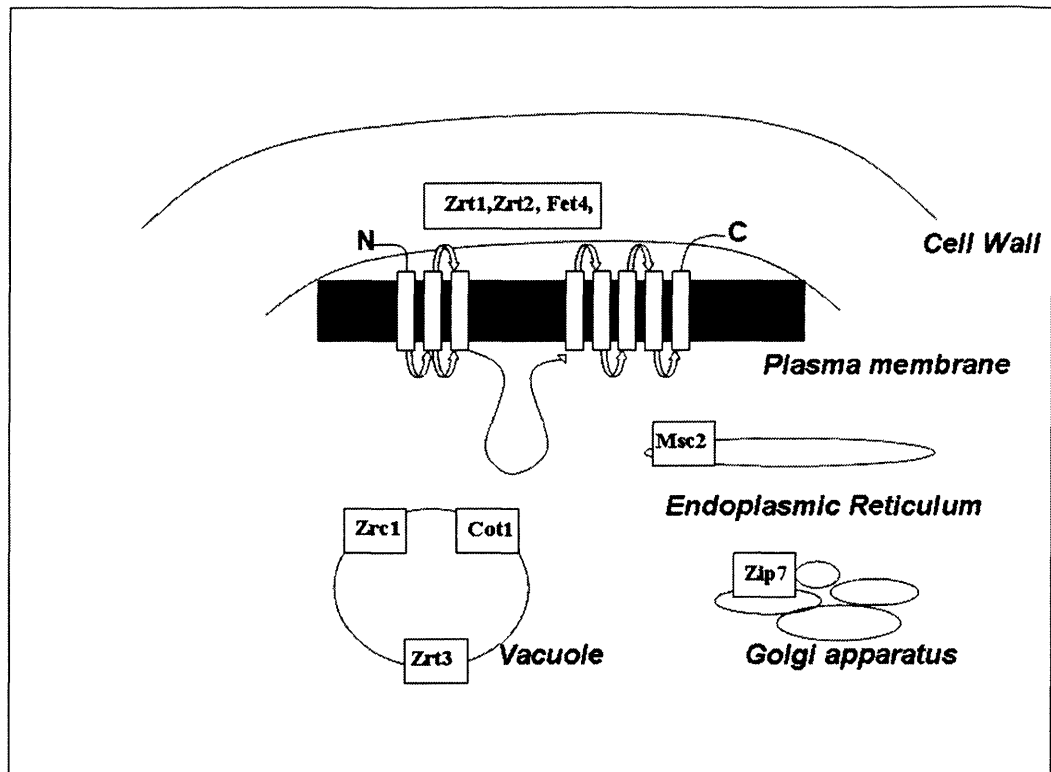
ZRT1 and *ZRT2* are the genes encoding for Zrt1 and Zrt2, respectively and *FET4* for the transporter fet4. The *ZAP1* gene, through its encoded activator Zap1, strictly controls the functioning of these genes and its own functioning, thanks to a mechanism of auto-regulation. Any controlled gene has one or more responsive elements (ZRE) in their promoters. Zap1 binds to ZRE to maximise the expression of the target genes. Zinc levels play an important role in this mechanism. For example, under severe zinc deficiency, Zap1 is produced at high levels and the affinity for the *ZRT1* is kept very high. The affinity for *ZRT2* is reduced, maybe because other proteins bind with the promoter of this gene, which consequently becomes unavailable for Zap1 (Eide, 2003). At post-translational level, zinc uptake is controlled by a mechanism of degradation of the protein Zrt1. At high zinc concentration for example, Zrt1 is transferred to the vacuole and inactivated through a mechanism of endocytosis and degradation (Gitan *et al.*, 1998).

A summary of the zinc transporters is reported in Table 1.10. Their localisation is depicted in Figure 1.2.

Table 1.10 Summary of main transporters involved in zinc homeostasis.

Transporter protein	Transporter Family	Zinc cell status
Zrt1	ZIP	Depleted
Zrt2	ZIP	Repleted
Fet4	ZIP	Normal
Zrt3	ZIP	From repleted to depleted
Zrc1	CDF	From depleted to repleted
Cot1	CDF	From depleted to repleted
Msc2	CDF	Normal
Zip7	ZIP	Depleted

Figure 1.2 Localisation of the main zinc transporters in the yeast cell. Information from Li and Kaplan (1998a), Miyabe *et al.* (2001), MacDiarmid, *et al.* (2000, 2002), Waters and Eide, 2002, Eide (2003), Ellis *et al.* (2004), Huang, *et al.* (2005) and relates to *S. cerevisiae*. See text for further information.



1.5.3 Zinc accumulation and detoxification by yeast

Once zinc is taken up into the cell, this metal is utilised for metabolic functions in the cytoplasm and in several organelles including mitochondria and endoplasmic reticulum. If zinc exceeds the requirements needed by the yeast cells, several mechanisms may be activated in order to store zinc until it is required or to detoxify it if zinc levels are above the toxicity threshold.

The yeast vacuole is known to accumulate several divalent cations, such as Zn^{2+} (White and Gadd, 1987), Ca^{2+} , Mn^{2+} , Fe^{2+} , Co^{2+} , Sr^{2+} , Ni^{2+} and the monovalent cations K^+ , Li^+ , and Cs^+ (Ramsay and Gadd, 1997; Okorokov *et al.*, 1985).

Even zinc transport into the vacuole is controlled at the transcriptional level. Three transporters are known to be localised in the vacuole: Zrc1 (Miyabe *et al.*, 2001), Cot1 (Li and Kaplan, 1998) and Zrt3 (MacDiarmid *et al.*, 2000, 2002). While the role of Zrc1 and Cot1 is to retain zinc brought in by endocytosis, the role of Zrt3 is to mobilise zinc out of the vacuole when zinc replete cells are exposed to zinc limiting

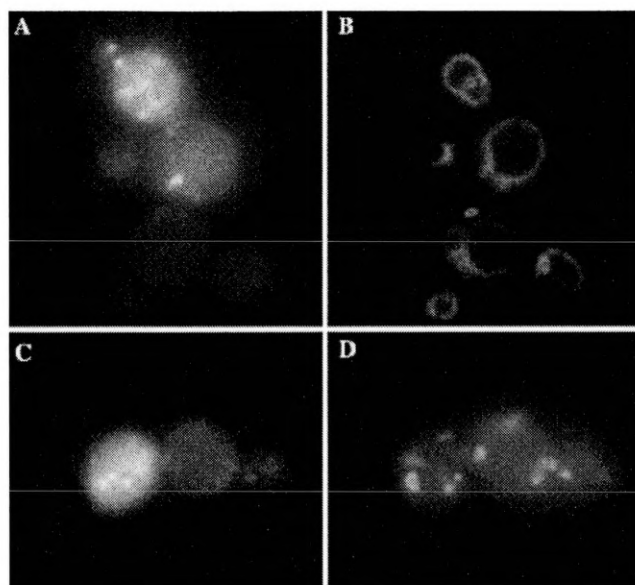
conditions. A fourth transporter Msc2, already known to influence zinc homeostasis (Li and Kaplan, 2001) has been recently localised in the endoplasmic reticulum where it is involved in supplying zinc to this compartment (Ellis *et al.*, 2004). More recently the transporter ZIP7, associated with the Golgi apparatus, has been found to have a role in zinc homeostasis, transporting zinc from the Golgi apparatus to the cytoplasm when yeast cells are grown in zinc depleted medium (Huang *et al.*, 2005).

When zinc deficient cells are suddenly exposed to high zinc concentrations, a “zinc-shock” occurs (MacDiarmid *et al.*, 2003). Zinc rapidly crosses the plasma membrane to accumulate in the cytoplasm first and then into the vacuole. As a consequence, the transcription of genes encoding for the plasma membrane transporters is down regulated and the Zrt1 protein is inactivated by endocytosis. This system prevents excessive zinc uptake. At the same time, the genes encoding for the vacuolar transporters are up regulated.

Studies with mutants have given very interesting information on the way zinc transport mechanisms act during zinc shock. Mutants lacking genes encoding for vacuolar transporters, and therefore unable to encode them, accumulate high cytoplasmic zinc concentrations which becomes so toxic that cell growth stops. The same mutants, lacking also the plasma membrane transporter Zrt1 (the high affinity system) have the capacity to grow. The plasma membrane and vacuole systems are continuously coordinated through the protein Zap1. Zap1 can interact with all the genes encoding for the plasma membrane transporters, *ZRT3* and *ZRC1* encoding for the vacuolar transporters but not with *COT1* encoding for the third vacuole transporter. Interestingly, Aft1 is the transcription factor that regulates *COT1* in conditions of iron deficiency. Most probably *COT1* is activated in order to suppress the accumulation of other unspecific metal ions that may accumulate in iron-limited cells (Foury and Talibi, 2001).

In *S. cerevisiae*, other systems have been described to play a role in zinc homeostasis. For example, Devirgiliis *et al.* (2004) have used specific fluorophores to detect zinc localisation and have been able to observe very small vesicles in the cytoplasmic periphery they named yeast *zincosomes* (Fig. 1.3). These vesicles appear very rapidly when zinc depleted cells are transferred to a medium containing micromolar zinc concentrations and disappear very slowly when the same cells are repitched in zinc-depleted medium. They may play a role as transporters of zinc ions to the sites quickly requiring this metal and to the vacuole to prevent toxicity.

Figure 1.3 Fluorescent staining of yeast *zincosomes*, vacuoles and mitochondria in wild type cells of *S. cerevisiae* Yeast cells were grown to logarithmic phase in SD medium and were stained with Zinquin (A) and with the vacuolar-specific fluorescent dye FM4-64 (B), or with Zinquin (C) and with the mitochondrial fluorescent stain Syto18 (D) (adapted from Devirgiliis *et al.*, 2004).



S. cerevisiae synthesises metallothioneins, low molecular weight, cysteine-rich proteins that bind heavy metals by means of their cysteine group, in order to sequester and detoxify metal ions (especially copper) when their concentration is in excess. No evidence of such a role in zinc sequestration has been shown in *S. cerevisiae* for these proteins (Palmiter, 1998) although in *Schizosaccharomyces pombe* the deletion of the gene *zym1*, encoding for one metallothionein, reduces zinc tolerance and the cell capacity to accumulate zinc (Borrelly *et al.*, 2002).

The high capacity of *S. cerevisiae* to sequester heavy metals like zinc, as well as uranium and copper, may be employed in bioremediation to decontaminate polluted industrial sites (Volesky and May-Phillips, 1995). A more comprehensive study has been recently published by Mapolelo *et al.* (2005) who studied the heavy metal uptake properties of various strains of *S. cerevisiae* in dam water, stream water, treated wastewater and industrial effluents. All the strains had high affinity for zinc but the uptake was pH dependent and influenced by the presence of other ions as Na^+ , K^+ , and Ca^{2+} that could suppress zinc uptake by 15-25%.

1.5.4 Zinc in industrial processes and in particular in brewing

In industrial fermentation media, several divalent cations such as zinc, magnesium, manganese, calcium are known to play important roles in yeast cell physiology and in dictating the progress of fermentation. Zinc media concentrations in the range 0.25-0.50 ppm have been reported to be optimal for cell growth, and 1-2 ppm for glycolysis (Jones and Greenfield, 1984). The concentration of this element is variable in different industrial media and the optimal zinc cellular level requirements are yeast strain dependent (Rees and Stewart, 1998). Part of zinc may also not be available (bio-available) to yeast cells because it is bound to some compounds present in the media or because it is present as zinc oxide and therefore is not taken up by yeast cells. This depends on the pH and the level of oxidation of the medium (Pourbuaix, 1963).

In the wine making industry, for example, zinc concentration in grape wine must ranges from 0.04 to 7.8 ppm and the average is 0.90 ppm (Cabanis and Flanzy, 1999). Usually in winemaking such zinc levels are satisfactory for the progress of fermentation.

In brewing, the mineral content in malt is usually 2-3% of the dry weight depending on the agronomic conditions and the trace element content of soil (zinc, manganese, iron, calcium and copper) which is usually about 0.02%. During wort preparation, a minor amount of metals is extracted, for example less than 5% of zinc, iron and strontium (Jacobsen and Lie, 1979; Jacobsen *et al.*, 1982). Jacobsen and Lie (1977) have analysed the degree of extraction of various elements and found an interesting correlation between zinc concentration in wort and the peptide and amino acid level, concluding that the cysteine groups may be active in the process of zinc sequestration. Other compounds are also known to take part to the process of ion chelation: phenols (Fe), α -amylase (Ca) and phytic acid (Ca, Zn and Fe).

The level of these compounds in wort may depend on the technical processes used during its preparation. Zinc levels usually decrease during the wort mashing, lautering and boiling (Daveloose, 1987; Jacobsen and Lie, 1977; Jacobsen *et al.*, 1982; Kreder, 1999). For example, a concentrated mash has a decreased protein level and consequently a lower level of zinc-binding compounds. During lautering or filtering of the mash, some ions may be washed out. In this respect, zinc ions become complexed in precipitated trub during the wort boiling and cooling and may become unavailable to yeast cells. Zinc binding with trub is loose. Kreder (1999) has demonstrated that keeping part of the trub into the wort during fermentation, is beneficial, because zinc

ions are slowly released into the medium. Since the trub has other nutritional and physical qualities, it is also needed for proper yeast growth and fermentation performance.

Yeast cells rapidly absorb zinc from wort, in the first hours after pitching and prior to the onset of fermentation (Mochaba *et al.* 1996; Bromberg *et al.*, 1997). Part of the zinc may be released and subsequently reabsorbed (Mochaba *et al.*, 1996). Various zinc concentrations do not usually influence cell growth, but this may be strain dependent (Rees and Stewart, 1998). Nevertheless, optimal zinc concentrations reduce the attenuation time considerably compared with zinc limited conditions (Skanks *et al.*, 1997). Sometimes the specific fermentation rate changes only after several successive fermentations (Bromberg *et al.* 1997).

Taylor and Orton (1973) demonstrated zinc to be an inhibitor of flocculation only at very high concentrations, above 6500 ppm and pH 7.5, concluding that zinc's flocculation role is unimportant in brewing as these conditions are unrealistic. An experiment *in vitro* showed that in terms of flocculation, lager strains are not affected by the presence of zinc, while some ale strains flocculated when the zinc concentration reached the so-called saturation point at 2.6 ppm (Raspor *et al.*, 1990). Above this level, de-flocculation occurred. This phenomenon may be explained by differences in the cell surface structures and may be employed to differentiate between ale and lager flocculating strains. In recent years, a study on yeast propagation demonstrated that only the addition of 0.30 ppm of zinc may have a consistent effect on flocculation (Wackerbauer *et al.*, 2004).

With regard to production of volatiles and higher alcohols by yeast, Skanks *et al.* (1997) have shown that zinc additions increased the levels of higher alcohols and esters but reduced acetaldehyde levels. Zinc preconditioned lager yeast cells produce distillates with higher alcohols (2-methyl-1-butanol, 3-methyl-1-butanol and isobutanol), esters and aldehydes and without iso-amyl acetate (Melville, 2003). Although addition of 0.5 ppm of zinc increased volatile organic compounds, it may also increase the concentration of medium chain fatty acids (MCFA) responsible for the unwanted soapy, fatty and rancid taste (Villa *et al.*, 1999).

Zinc deficiencies during fermentation may cause serious problems in terms of diminished yeast cell growth during propagation and in terms of reduced ethanol production. Generally speaking, when zinc levels fall below around 0.1 ppm, then fermentation problems may be encountered (Helin and Slaughter, 1977b; Jacobsen & Volden, 1981; Bromberg *et al.*, 1997) and this may lead to slow and incomplete

fermentations, which are termed “sluggish” (Jacobsen *et al.*, 1982). This may be explained by the reduced activity of the metallo-enzyme alcohol dehydrogenase.

Excess of zinc in wort could be toxic but this seems to be dependent on the presence of manganese. Helin and Slaughter (1977b) observed that only 0.6 ppm of zinc are toxic if Mn level is below 0.01 ppm. Jones and Greenfield (1984) reported that only 2 ppm zinc can be tolerated if Mn levels are less than 0.4 ppm. This limit is extended to 65.5 ppm if Mn is above 0.4 ppm. It is clear that it is very difficult to determine the limits of zinc toxicity since this may depend on the interaction with other elements. Generally speaking, in the range of 5-50 ppm, several divalent cations influence the uptake of other divalent cations (Helin and Slaughter, 1977b). In relation to other ions, zinc is known to have the following interaction properties (Jones and Greenfield, 1984):

- antagonism of Cu^{2+} and Cd^{2+} toxicity.
- enhancement of the beneficial growth properties of Ca^{2+} and Mn^{2+} .
- Cu^{2+} level stimulation of Zn^{2+} absorption.
- stimulation of growth in communion with Mn^{2+} and Cu^{2+} .

High gravity wort may help yeast cells to tolerate extremely high zinc concentrations up to 1310 ppm. This effect has been demonstrated in some lager yeast cells that keep the same attenuation time and maintain a relatively good viability (about 50%) compared to the viability they would have in normal gravity worts. This may depend on the different tolerance to zinc of various strains as well as the greater buffering capacity of high gravity wort (Rees and Stewart, 1998).

Zinc interaction with yeast, its transport and utilisation, during brewing and other industrial processes, are phenomena still not completely understood.

To date, most of the efforts of the applied research were concentrated towards the determination of zinc bio-availability in malt wort and the discovery of new technologies to improve such availability. Studies on zinc uptake kinetics were merely aimed to describe variation on zinc wort levels during fermentation and to determine the zinc concentration for the best fermentative performance.

In the last ten years *S. cerevisiae* has been employed as a model in biomedical studies to understand zinc homeostasis, to analyse and describe the transport systems and related encoding genes involved in zinc uptake, accumulation and detoxification. Unfortunately, in recent years, few studies have been carried on to elucidate the fascinating interaction of zinc with industrial yeast strains and to answer crucial

questions related to the kinetics of zinc uptake, the localisation of zinc into the cell and its population distribution. Although various studies have been conducted to determine the antioxidant effects of zinc, it is not clear if zinc may help yeast cells to alleviate the effects of some physical and chemical stresses encountered in industrial fermentation environments. Despite all the studies conducted to determine the genes involved in the transport of zinc into the cell and in various cell compartments, no definitive study has yet been carried out to use these findings for determining the status of cellular zinc in yeast.

1.6 Aims and objectives of this Thesis

The central role of metal ions, and in particular of zinc, on yeast cell physiology is a phenomenon known but not completely understood. In recent years the yeast-based industries have shown an increased interest to optimise metal ion bio-availability and improve yeast fermentation performance.

In order to fulfil the lack of knowledge on zinc interaction with yeast cells and in order to respond to the demand of how this could be usefully applied to the correct management of zinc in industrial fermentation processes, the aim of this project was to generate fundamental information on how zinc affects yeast physiology. Increased bio-availability of this metal, improvement of yeast growth, viability, fermentation performance, flavour profile and resistance to environmental stresses represent some of the applied aims of this research.

The present Thesis is divided in separate chapters. The research described herein may be considered interdependent. Specific objectives of each Chapter were as follows:

Chapter 3: Zinc uptake by industrial yeast strains:

- To describe zinc uptake kinetics by various industrial yeast strains in laboratory scale experiments.
- To describe zinc uptake kinetics under various conditions including different temperatures, various zinc concentrations and salt sources.
- To determine the metabolic mechanisms occurring during zinc uptake for a better understanding of the influence of cell status on zinc uptake.

Chapter 4: Cellular localisation and population distribution of zinc by a brewing yeast strain:

- To understand the mechanisms related to the accumulation of this ion.
- To visualise free intracellular zinc using novel methods based on microscopy and flow cytometry.
- To describe the proportion of zinc shared between mother and daughter cells during cell division in order to provide information for the brewing industry where yeasts are repitched for several fermentations.

Chapter 5: Influence of zinc on brewing yeast fermentation performance:

- To examine the influence of zinc on yeast fermentation performance in simulated small-scale industrial experiments, in wine making and brewing.
- To investigate the interaction of various zinc levels on fermentation performance and beer flavour profiles in a brewery pilot plant.

Chapter 6: Influence of physical and chemical stresses on zinc homeostasis in brewing yeast:

- To analyse the effect of various industrial stresses on yeast cells in terms of cellular zinc content.
- To analyse the possible role of the plasma membrane on the cellular zinc content during such stresses.

Chapter 7: Studies on zinc limited growth of *S. cerevisiae*:

- To define the effect of zinc deficiency on yeast fermentative performance in both batch and chemostat continuous cultures.
- To determine at the transcriptome level, the genes involved during zinc limitation.
- To determine a biomarker available to the industry able to determine the condition of cellular zinc limitation.

Chapter 2

General materials and methods

2.1 Microorganisms used and culture maintenance

The yeast strains of *Saccharomyces cerevisiae* employed in this work are listed in table 2.1

Table 2.1 Yeast strains used in this Thesis (all *S. cerevisiae*)

Yeast strain	Code	Source
Wine strain (Wine A)	L-2056	Lallemand Inc., Montreal (Canada) courtesy of Mr. J. McLaren
Wine strain (Wine B)	L-2226	Lallemand Inc., Montreal (Canada) courtesy of Mr. J. McLaren
Lager brewing strain (Lager A)	McL	Scottish Courage Brewing, Edinburgh (UK), courtesy of Dr. B. Taidi
Lager brewing strain (Lager B)	LMA	Heineken Supply Chain, Zoeterwoude (NL), courtesy of Dr. M. Walsh
Lager brewing strain (Lager C)	LMB	Heineken Supply Chain, Zoeterwoude (NL), courtesy of Dr. M. Walsh
Ale brewing strain	NCYC 1681	Brewing Research International, (UK). courtesy of Dr. J. Hammond
Whisky strain	DCLM	Quest International, Menstrie (UK), courtesy of Dr. I. Maynard
Baking strain	GB4918	Quest International, Menstrie (UK), courtesy of Dr. I. Maynard
Laboratory strain (wild type haploid)	CEN.PK113- 7D	Technical University of TUDelft, Delft (NL), courtesy of Prof. J. Pronk

Note: one strain of *Kluyveromyces marxianus* (NCYC1425) and one of *Schizosaccharomyces pombe* (DBVPG1354) were also used for zinc uptake preliminary studies.

Yeast cultures were maintained at -80°C, according to the following procedure. Cells were grown up in shake flasks, in 2% malt extract broth (refer to paragraph 2.2.2) at 25°C for 48 hours and checked for number, viability and contamination. A glycerol solution was added to a final concentration of 20% and the suspension was transferred aseptically to sterile cryo-vials. Cell suspensions were transferred to a freezer at -20°C for 2 hours and then to a freezer at -80°C until required.

When needed, cultures were thawed at room temperature and a loopful of cells was transferred aseptically, to malt extract agar slopes, in triplicate. After 48 hours of growth at 25°C, slopes were transferred in refrigerator at 5°C and stored for 1 month.

2.2 Inocula preparation and yeast culture conditions

2.2.1 Inocula preparation

Seed cultures were prepared aseptically by inoculating a loopful of cells from refrigerated slope cultures into liquid media. After 24 hours of growth, a calculated amount of seed culture volume was centrifuged and cells washed once with sterile deionised water and resuspended in experimental medium, according to the following calculation:

$$V_1 = \frac{V_2 \times N_2}{N_1}$$

V_1 = inoculum volume.

V_2 = experimental medium volume.

N_1 = cell number /ml in seed culture.

N_2 = desired cell number/ml in experimental culture ($5 \times 10^6 - 10^7$ cells/ml).

2.2.2. Yeast growth media

Malt extract broth 2 % (from Oxoid Ltd, UK) medium was prepared according to the instructions provided by the company. In experiments of zinc uptake by dead cells and in the use of protein synthesis inhibited cells by cycloheximide, maltose was added to increase the specific gravity of the medium.

Malt wort was prepared in the laboratory preparing a 25% (w/v) suspension of barley malt grist (Optique variety, kindly provided by Baird Malt, Arbroath, UK) in pre-heated (65°C) deionised water. This mash was kept at 65°C for 1 hour to allow the conversion of starch into maltose, which was checked using an iodine solution. The final malt wort was filtered, autoclaved at 120°C for 20 min and clarified by centrifugation in order to separate precipitated proteins. Analyses of malt wort were carried out to determine original gravity (OG), pH, zinc levels, and when required, magnesium and calcium concentrations.

Malt wort used in pilot plant experiments was kindly prepared by the Pilot Plant staff of Heineken Supply Chain, Zoeterwoude, the Netherlands. The medium was supplemented with hop extracts and pre-aerated before inoculation. The original gravity was 1057 (14°P) and the level of zinc was below the limit of detection of the

Atomic Absorption Spectroscopy used for the measurements of the cations (0.10 ppm). In discussion, the medium without added zinc will be referred to as “zinc free”.

Grape juice medium (sugar concentration 15%) used in wine-making experiments was purchased by Welch Ltd and centrifuged at 1500 g at 5°C for 10 minutes to remove large size particles.

A chemically defined synthetic medium Edinburgh Minimal Medium number 3 (EMM3) (Table 2.2), was prepared in order to simulate a malt wort medium (1023°OG), by modifying EMM3 of Fantes and Nurse (1977), according to the method of Chandrasena (1996).

Table 2.2 Composition of EMM3 (synthetic brewers' wort)

Ingredient	amount/Lt
Glucose	10 g
Fructose	3.3 g
Sucrose	5.3 g
Maltose	40 g
Ammonium Sulphate	5 g
Sodium Hydrogen Phosphate	1.42 g
Magnesium sulphate 7 H ₂ O	2.46 g
Potassium Hydrogen Phthalate	3.06 g
Calcium Chloride	10 mg
Potassium Chloride	1 g
Di-Sodium Sulphate	300 mg
Trace-Elements:	
Boric acid	0.50 mg
Manganese Sulphate	0.40 mg
Ferric Chloride	0.20 mg
Molybdic acid	0.16 mg
Potassium Iodide	0.10 mg
Copper Sulphate	0.04 mg
Citric acid	1.0 mg
Vitamins:	
Inositol	10 mg
Nicotinic acid	10 mg
Calcium Pantothenate	1.0 mg
Biotin	0.01 mg

Vitamins and trace-elements solutions were prepared at 1×10^3 concentrated stock solutions, sterilised by filtration and added in calculated amounts to the medium, after autoclave sterilisation, to reconstitute the normal concentration.

The medium was unsupplemented with zinc. A zinc sterile solution was added in calculated amounts to adjust zinc levels, when required, only prior to the beginning of the experiments.

EMM4 synthetic wort medium was a variation of EMM3, with an increased maltose concentration of 115.5 g/Lt, according to the level proposed by Kennedy *et al.* (1997) to have an initial OG of 1055 (13.5°P). The grade of the supplemented maltose contained up to 7% maltotriose, which was representative of a typical brewery malt wort.

Defined synthetic medium (Table 2.3) for chemostat continuous cultivation of yeast was prepared according to the method of Verduyn *et al.* (1992).

Table 2.3 Composition of minimal medium for chemostat continuous cultures

Ingredient	ml/Lt	Amount/Lt
Ammonium Sulphate		5.0 g
Potassium Hydrogen Phosphate		3.0 g
Magnesium sulphate 7 H ₂ O		0.5 g
Glucose		57.55 g
Silicone antifoaming agent	0.15	
Tween/ergosterol solution	1.25	
Trace-Elements:*		
EDTA (Titrplex III)		0.15 mg
Manganese Chloride Dehydrate		0.84 mg
Cobalt Chloride Hexahydrate		0.30 mg
Copper Sulphate Pentahydrate		0.30 mg
di-sodium Molybdenum Dihydrate		0.40 mg
Calcium Chloride Dihydrate		4.50 mg
Iron Sulphate Heptadihydrate		3.00 mg
Boric acid		1.00 mg
Potassium Iodide		0.10 mg
Biotin (D-)		0.05 mg
Ca D(+) Panthotenate		1.00 mg
Nicotinic acid		1.00 mg
Myo-Inositol		25.00 mg
Thiamine Chloride Hydrochloride		1.00 mg
Pyridoxol Hydrochloride		1.00 mg
p-Aminobenzoic acid		0.20 mg

Tween-ergosterol solution:

Ingredient	ml	Gram
Ethanol	17	
Tween 80		8.4
Ergosterol		0.2

Ammonium sulphate, potassium hydrogen phosphate, magnesium sulphate 7 H₂O, trace element solution (from a 1000x concentrated stock solution) and silicone antifoaming agent were added together prior to autoclaving. Glucose solution was prepared as a 50% (w/v) solution, autoclaved separately and added aseptically together with the vitamins (from a 1000x concentrated stock solution), after cooling of the previous solution. In anaerobic experiments, tween-ergosterol was added to the medium because yeast cells are not able to metabolise this compound in oxygen absence. Since ergosterol is sensitive to light, the medium reservoir vessel was covered with a black foil to protect it. The tween-ergosterol solution was not added to the aerobic chemostat continuous culture.

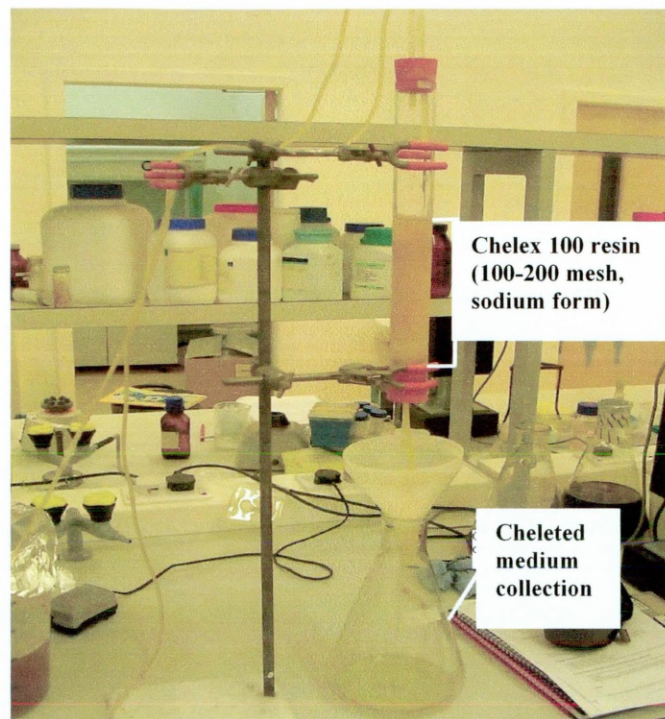
2.2.3. Zinc media depletion by biochelation, chelation and zinc level media adjustments

In experiments with zinc-depleted batch cultures, medium EMM4 was made zinc free through the following process of “biochelation”. Yeast cells were inoculated in the medium at an initial cell density of 5×10^6 cells/ml and kept in suspension for 1 hour at 25°C. These conditions showed (see chapter 3) that yeasts removed all zinc from their growth medium. Cells were centrifuged at 5°C, 1100 g for 5 minutes and the supernatant poured into a sterile filter (Whatman, cellulose acetate, 0.45 µm) placed onto a sterile and deionised (see paragraph 2.2.3) Buchner-flask with filtering device connected to a vacuum pump. Such a medium was assumed to be normal in all aspects except zinc levels.

In the simulated brewing fermentation experiments in Imhoff conical vessels (Chapter 5), malt wort (OG 1055, Mg at 146 ppm, Ca at 20 ppm, Mn at 20 ppm) was depleted of zinc using the same procedure described above with the exception of the centrifuge process, for which a continuous centrifuge was employed (5°C, 12000 rpm).

In experiments on the influence of calcium on zinc uptake, malt wort was made free from divalent cations using a deionised column (Fig. 2.1) constructed using Chelex® 100 resin (100-200 mesh, sodium form, from BioRad) as the chelating agent. The medium was fed into the column via a peristaltic pump, to help create pressure to pass the wort through. The column was filled with 5g of the resin per 100ml of medium. With 100g this gave the column a potential capacity of 2 litres before it would need to be regenerated.

Figure 2.1 De-ionisation column construction using Chelex®.



The column was regenerated using the following procedure:

- Washing with 2 bed volumes of 1N HCl
- Washing with 5 bed volumes of deionised water
- Washing with 2 bed volumes of 1N NaOH
- Final washing with deionised water to reduce the pH (the pH will never fully return to a neutral pH. Therefore, the final medium pH was altered using phosphoric acid).

Malt wort was passed through the column a second time to completely remove trace amounts of zinc. The medium pH was finally corrected to 5.2 and divalent cations, except zinc, replenished to original levels before being autoclaved.

Zinc concentrations were altered adding calculated volumes of a 1000 ppm zinc acetate stock solution (for shake flask and conical vessel experiments) or zinc sulphate salt (for pilot plant experiments).

2.2.4. Glassware and fermenter acid wash

Glassware, flasks and fermenters employed in this study were all washed to remove any metal cation contaminations, including zinc, according to the following procedure: overnight soaking in 2% nitric acid, two washes with deionised water, one wash with 0.1 M EDTA, four washes with deionised water and final drying. This procedure was not employed in the pilot plant experiment because of technical difficulties due to the large size of the fermenters employed in this study.

2.2.5. Growth conditions

Shake flask experiments were conducted using an orbital incubator at 200 rpm, at a constant temperature of 25°C. Yeasts were inoculated at initial cell densities of 5×10^6 cells/ml.

Conical Imhoff vessels (volume 1L, cone angle 74°), were employed in small-scale brewing and wine fermentation experiments (Fig. 2.2). In brewing experiments, malt wort was added with isomerised hop extracts pre-aerated at 14°C for 2 hours using filtered air and yeast cells inoculated at 5×10^6 cells/ml. Fermentation was carried out for 11 days at 14°C and samples taken every 2 days. In wine-making experiments, yeast cells were inoculated into grape juice at 12×10^6 cells/ml and fermentation was carried out for 5 days at 25°C.

Brewery pilot plant experiments were performed at Heineken Supply Chain, Zoeterwoude, the Netherlands, in 200 Lt fermenters (Fig 2.3) with a cone angle between 60 and 70°. Pre-aerated hopped malt wort was poured into the fermenters using a system of tubes directly connected to the malt wort preparation vessel. Yeast cells were inoculated at approximately 10×10^6 cells/ml initial cell number. Inoculum was added from the top of the fermenter under flame to avoid contamination. This operation was done during the malt wort filling process to allow an initial homogenous mixing of the seed culture. Fermentation was carried out for 8 days at 11°C. Although the fermenters were not sterilised prior to inoculum, no major contaminations were observed during the fermentation.

2.3 Zinc limited yeast culture conditions

Yeast seed cultures were prepared in shake flasks containing 100 ml of the same minimal medium used for chemostat continuous cultivation, with a glucose concentration of 2%, without ergosterol solution and free of zinc. Inocula were prepared aseptically adding a cryovial of the *S. cerevisiae* strain CEN.PK113-7D

previously depleted of zinc (grown for 24 hours in a series of four subsequent flask cultures in zinc free media). Seed cultures were grown in an orbital incubator at 30°C, 200 rpm for 24 hours.

Continuous cultures were established in 2 L Applikon chemostats (Fig. 2.4) with a working volume of 1.0 L filled with 900 ml of minimal medium for continuous cultivation, inoculated with the seed culture described above. Physical conditions were as follows: pH 5.0, temperature 30°C, stirrer 800 rpm, gas flow 500 (± 10) ml/min. Oxygen, pH and base were monitored using an online system, Applikon ADI 1030 Biocontroller. After an initial phase when yeast cells were cultivated in batch phase and without the activation of the nutrient feed pumps, continuous cultures were started when all glucose and ethanol were consumed and the pH showed a small increase, generally after 15 hours. The dilution rate was set at 0.10 h^{-1} . The pH was measured online and kept constant at 5.0 by the automatic addition of 2 M KOH with the use of the biocontroller. Anaerobic conditions were maintained by sparging the medium reservoir and the fermenter with pure nitrogen gas ($0.5 \text{ liter} \cdot \text{min}^{-1}$). Aerobic conditions were maintained by sparging pure air into the fermenter. The off-gas was cooled by a condenser connected to a cryostat set at 2°C. Oxygen and carbon dioxide were measured offline with a NGA 2000 Rosemount gas analyser. After 4 volume changes (40 hours of growth), pre-steady state samples were taken for: yeast dry weight, glucose concentration, temperature, gas flow, pressure and temperature. Steady-state samples were taken after approximately 10 volume changes from the beginning of the continuous culture, to avoid strain adaptation due to long term cultivation. When biomass dry weight, metabolite and gas profiles were constant over at least 3 volume changes, the culture was considered in steady-state.

Initial glucose levels were calculated to leave a final residual glucose in the range 17-21 g/L. This condition was essential to make comparisons between zinc limited and nitrogen limited chemostat continuous cultures.

Figure 2.2 Conical vessels (1L) used for simulated brewing (a) and wine-making (b) experiments

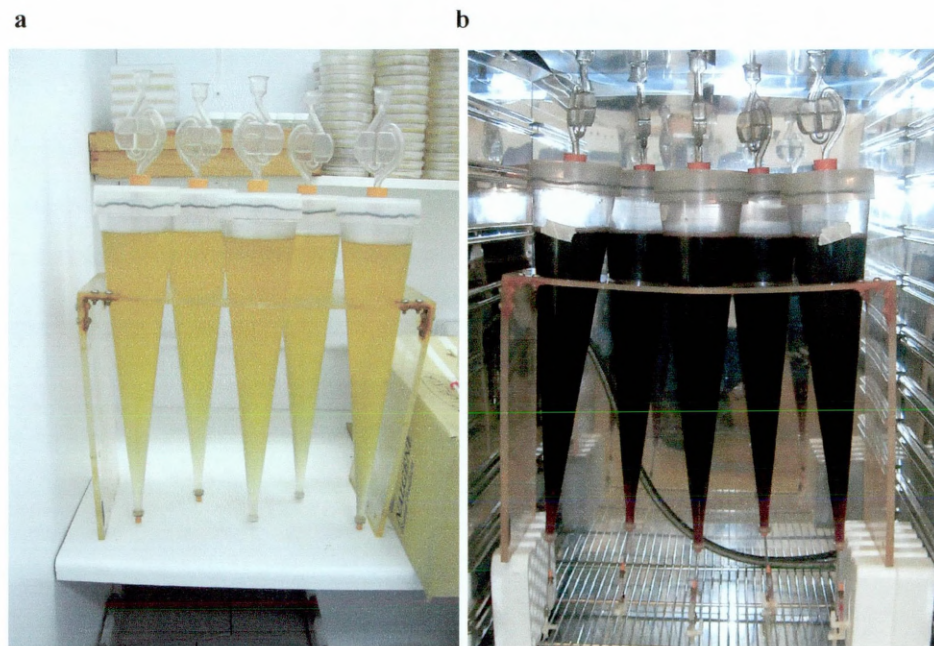
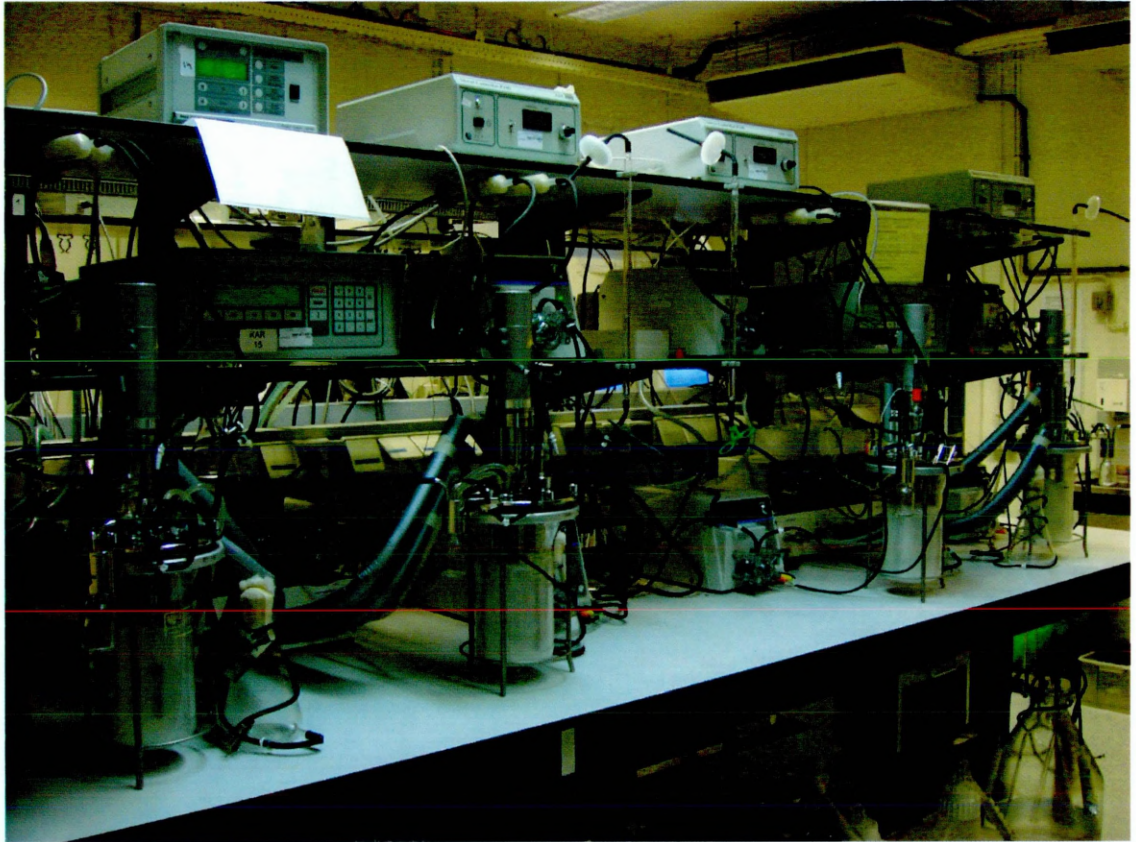


Figure 2.3 Brewing pilot plant fermenter (200 L)

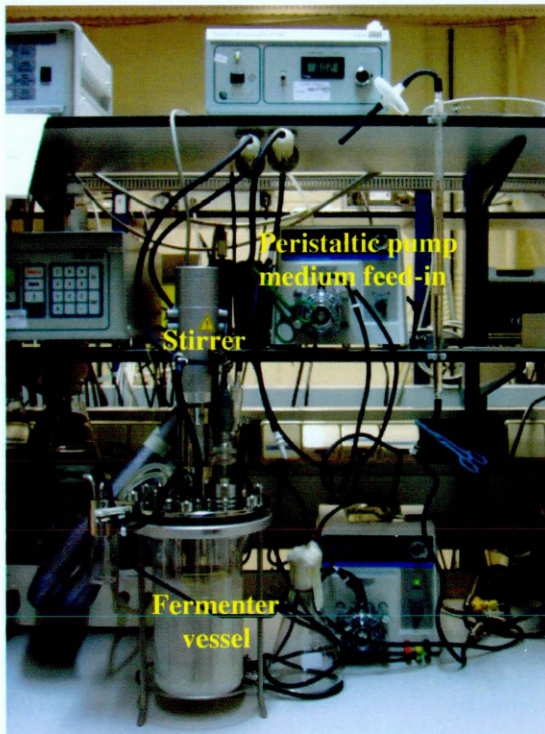


Figure 2.4 Chemostat continuous culture fermenters. A group of three fermenters were used to get three independent replicates of the same condition (a). Location of the fermenter vessels and of the medium reservoir and waste are shown respectively in figure b and c. Chemostat experiments were conducted at TUDelft (the Netherlands).

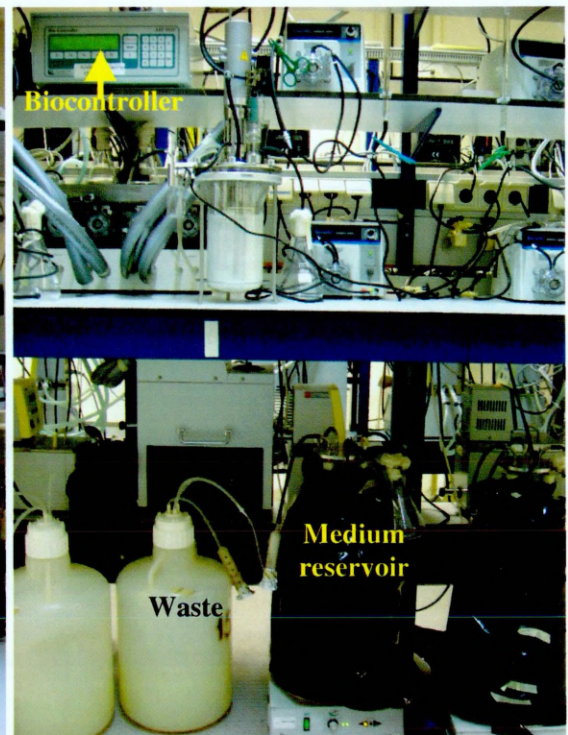
a



b



c



2.4 Determination of yeast growth

Yeast cell number was determined using a Haemocytometer (Neubauer Improved), with a bright field microscope (400x magnification).

In the pilot plant research, cell number was determined using a Coulter Multisizer II after suitable dilution in isotonic solution (0.9% Na Cl).

Yeast dry weight was assessed according to the method of Postma *et al.* (1989a). 10 ml of the culture sample was poured into a pre-weighted membrane filter (Whatman, cellulose acetate, 0.45 µm) on a Buchner-flask with filtering device connected to a vacuum pump, using a calibrated pipette. The pre-weighted filter was washed once with 10 ml of deionised water and placed in microwave at 360 watts for 20 minutes. The filter was dried in a desiccator for 30 minutes and weighed. The biomass was calculated by difference and referred as g/L. Each sample was analysed in duplicate.

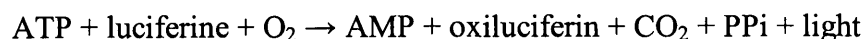
Yeast cell viability was evaluated using a Methylene Violet solution (Smart *et al.*, 1999): 2% (w/v) Methylene Violet 3 Rax (Sigma) in sodium citrate 0.01%. Cell suspension, diluted when necessary, was resuspended in 1:1 methylene violet solution. Dead cells were stained violet.

In the brewing pilot plant experiment, viability was checked using a Methylene Blue (BDH Chemicals Ltd, Poole, England) solution using the same preparation described above except that Methylene Violet was replaced by the Methylene Blue.

2.5 Biochemical assays

2.5.1 ATP assay

The assay was adapted from the method of Wishart (1982). Yeast cells were centrifuged, washed twice in dH₂O and reconstituted in 1 ml of dH₂O. 100 µL of this suspension was injected into 200 µL of pre-heated dH₂O at 95°C for 2 minutes and left for further 2 minutes at 95°C to allow complete cell digestion. Cells were spun down and supernatants containing the released ATP were kept for the assay. ATP can be easily detected using the phenomenon of bioluminescence (Briggs *et al.*, 2004). The firefly, *Photinus pyralis* contains an enzyme, luciferase. In the presence of oxygen and ATP, this enzyme catalyses the following reaction:



Luciferine is oxidised to oxyluciferin, ATP is hydrolysed to ADP. Each mole of ATP hydrolysed produces a photon of light with an emission maximum of 532 nm. The assay was carried out using a luminometer (1250 LKB Wallac) connected to a potentiometer (Servogor 120 BBC Goerz Metrawatt). Calculated volumes of samples

containing ATP were mixed with Luciferase (Sigma) and the intensity of the light emitted by the reaction of the two compounds was analysed using the luminometer, at 10 mV or 1V sensitivity. The instrument was previously calibrated using ATP standards (Sigma) in the range 0.1-10 μ M.

2.5.2 Zinc and vacuole staining

The following procedure was used to visualise cellular zinc using a fluorescent method. Yeast cells were spun down, resuspended and washed thrice in PBS. The pellet was finally resuspended at a concentration of approximately 5×10^7 cells/ml in a solution of PBS with 10 μ M Fluo-Zn3 (Molecular Probes), diluted from a 1 mM stock solution in DMSO. Cells were kept shaken in incubator at 30°C, 200 rpm for 1 hour (to allow complete uptake of the probe) and washed three times in PBS. The suspension was kept shaken in PBS for further 30 minutes to allow complete de-esterification of the probe and allow the active portion of the fluorophore to bind free zinc ions.

The following procedure was used to visualise yeast vacuoles. Cells, pre-stained with Fluo-Zn3, were washed twice in PBS and a solution of PBS with 10 μ M Cell-Tracker B (Molecular Probes) was added from a 1 mM stock solution in DMSO. Cells were kept shaken for further 30 minutes at 25°C and eventually washed three times in PBS prior to visualisation using a microscope Leica DMR. Controls were prepared using the same procedure but excluding the probes from the PBS buffers. Yeast cells analysed by flow cytometry were stained with 10 μ M Rhod-Zn1 in PBS using the same procedure described above with the exclusion of the vacuole staining. Cells were resuspended at the same concentration prior to flow cytometer analyses, using a Partec Cyflow.

2.5.3 Membrane fluidity assay

The fluorescent membrane probe Laurdan (Molecular Probes, 6-lauroyl-2-dimethylamino naphthalene) was used. This fluorophore exhibits a 50 nm red shift in emission spectrum over the gel to liquid crystalline phase transition of lipid bilayer systems. The Generalised Polarization (GP) parameter is used as an index of membrane fluidity. GP is determined from relative fluorescence intensities at wavelengths representing gel and liquid crystalline phases, using the following equation (Parasassi *et al.*, 1990):

$$GP = \frac{I_{gel} - I_{lc}}{I_{gel} + I_{lc}}$$

Where I_{gel} and I_{lc} indicate relative fluorescence intensities at wavelengths representing gel (440nm) and liquid crystalline (490nm) phases of bilayer systems, respectively. Cells to be analysed were centrifuged, washed with dH_2O , resuspended in dH_2O at $0.1 OD_{600\text{ nm}}$, and labelled by mixing with laurdan stock in ethanol (1 μL per mL cells, final laurdan concentration 5 μM) and incubating for 1 h in the dark. This negligible addition of ethanol was previously shown not to affect yeast. GP of Laurdan was measured as previously described (Learmonth and Gratton, 2002) using a Perkin-Elmer LS-3B Fluorescence Spectrometer.

2.6 Chemical assays

2.6.1 Cell and supernatant digestion

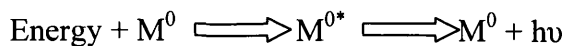
Yeast cells in the range 2×10^7 - 2×10^8 cells/ml were centrifuged and the pellet was washed thrice in deionised water, resuspended in 1 ml concentrated nitric acid (69% AnaLar) and heated in a water bath at 90°C for 1 hour to allow complete cell hydrolysis (evident by a clear hydrolysate solution). This solution was diluted with dH_2O prior to AAS analysis.

Malt wort supernatant was also collected and detected by AAS after acid digestion. This operation was required to digest malt wort proteins which may have bound part of the zinc ions. The sample was diluted 1:1 with nitric acid and dH_2O was added to obtain a final 20% sample solution. Prior to Ca analyses, Lanthanum chloride was added to the samples to a final concentration of 0.2% to avoid matrix interferences depending on phosphates. Samples were analysed by AAS and dilutions were made up when the zinc concentrations in samples were above the AAS calibration range (0 to 1 ppm). Each sample was analysed in triplicate.

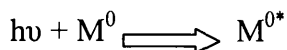
2.6.2 Atomic Absorption Spectroscopy (AAS)

Samples were analysed for zinc, magnesium and calcium by Atomic Absorption Spectroscopy. Heating vaporised the sample into free atoms and a certain wavelength of light, characteristic for each element, was passed through the vapour. As the amount of ground state atoms in the path increased, the amount of light absorbed also increased so that measurements of light absorbed permitted a quantitative determination of the amount of analyte present (Sanui and Rubin, 1982).

a) Excitation and emission



b) Atomic absorption



M^0 = Ground state element

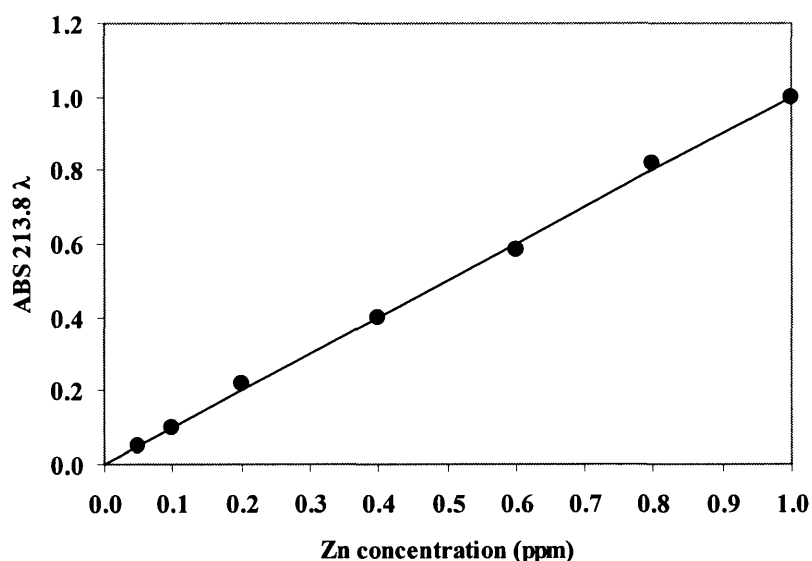
M^{0*} = Excited state element

$h\nu$ = Light energy

Since the wavelength absorbed is specific for each element, no interference occurred with other elements. Matrix interferences could occur if the sample detected is rich in dissolved solutes. Thus, dilution of samples was necessary to minimise such error.

The concentration of the elements in the sample was determined by comparison with the calibration curve obtained by flame atomisation of the following seven standards for zinc, calcium, magnesium and manganese analyses: 0.05, 0.10, 0.20, 0.40, 0.60, 0.80, 1.0 ppm (Fig. 2.5).

Figure 2.5 AAS calibration curve for zinc analyses



The fuel used for flame atomisation of each element was acetylene (C_2H_2) with a flow rate of 2.5 ml/min. The oxidant used was air with a flow rate of 8.0 L/min. The programs used for each metal are reported in Table 2.4

Table 2.4 Program set up for element analyses by AAS

	Zinc	Magnesium	Calcium	Manganese
Lamp	<i>Zn</i>	<i>Ca/Mg</i>	<i>Ca/Mg</i>	<i>Mn</i>
Current	<i>15 mA</i>	<i>12 mA</i>	<i>12 mA</i>	<i>15</i>
Wavelength	<i>213.8 nm</i>	<i>285.2 nm</i>	<i>422.7 nm</i>	<i>279.2 nm</i>
Slit	<i>0.7 nm</i>	<i>0.7 nm</i>	<i>0.7 nm</i>	<i>0.2nm</i>
Energy	<i>41</i>	<i>72</i>	<i>72</i>	<i>41</i>
Background correction	<i>ON</i>	<i>ON</i>	<i>OFF</i>	<i>ON</i>
Calibration	<i>Linear AA-BG</i>	<i>Linear AA-BG</i>	<i>Linear AA</i>	<i>Linear AA-BG</i>

2.6.3 Ethanol, sugar and metabolite analyses

In the conical vessel fermentation experiments, ethanol levels were analysed using a Gas Chromatograph Mass Spectrometer GCMS-QP2010 fitted with a Agilent HP Blood Alcohol capillary column (ID: 0.32 mm, length: 7.5 m, film: 20 µm). Sugar concentrations were analysed in wort by an Anton Parr DMA 58 Density Meter (courtesy of Baird Malt, Arbroath, UK).

In brewery pilot plant experiments, sugar levels were analysed by Density meter DMA48 (Anton Paar), combined with the Alcolyzer Plus (Anton Paar) for ethanol analyses.

The determination of esters and alcohols in green beer was obtained using headspace gas chromatography (GC). Separation of the various compounds was achieved on a Carbowax 400 or HP-Wax wide bore column. For detection, a Flame Ionisation Detector (FID) was used (courtesy of Quality Control, Heineken Supply Chain, the Netherlands).

In zinc limitation experiments, sugar, ethanol and metabolite analyses were carried out using high performance liquid chromatography (HPLC) fitted with an Aminex HPX-87H ion exchange column using 5 mM H₂SO₄ as the mobile phase.

2.7 Determination of degree of turbidity during fermentation (haze)

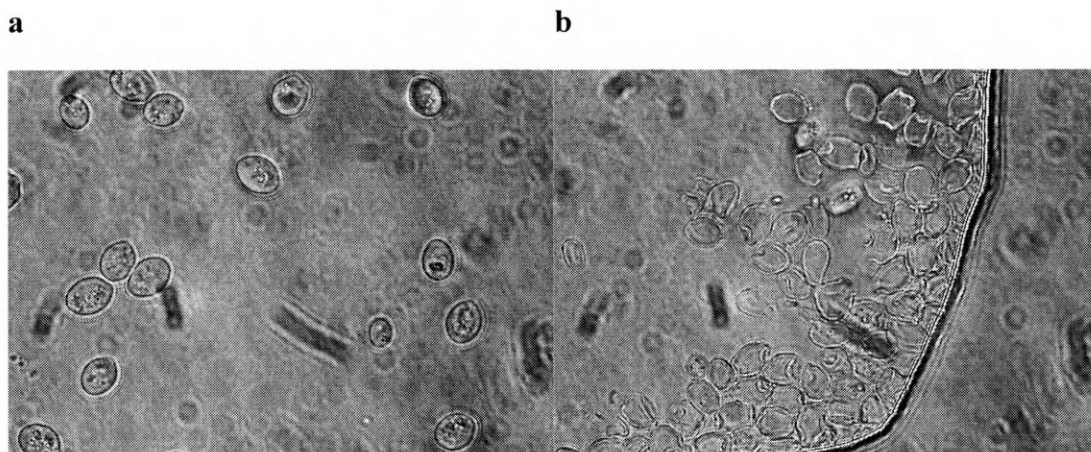
Cell suspensions were centrifuged at 2300 g for 7 minutes to remove cells and small particles. Supernatants were retained from shake flasks and degasified in an orbital shaker, at 200 rpm for 30 minutes. Samples were added with 1 gram of diatomaceous earth and passed through a membrane filter, 3 µm pore size and 47 mm diameter (Millipore) using a glass filter holder and vacuum pump. The filtrate was collected in a flask and analysed by turbidity meter Sigrist Photometer to measure scattered light (at

90° angle). This method was developed by the Heineken Supply Chain (Zoeterwoude, the Netherlands).

2.8 Preparation of yeast cell walls

Yeast cell samples were washed twice with dH₂O and treated with acid-washed glass beads (425-600 µm, 30-40 U.S. sieve, Sigma). Cells were agitated at high speed by vortex mixing, 12 times for 30 seconds each time. After each vortex, samples were kept on ice for 30 seconds to avoid cell overheating. Cell suspensions were finally separated from the glass beads by centrifugation (1500 g for 5 minutes, at 5°C) and washed 8 times with dH₂O to remove any protoplast fragments and any loosely bound zinc. As a result, suspensions of washed cell walls were obtained (Fig. 2.6). When a zinc chelator (0.01% 1:10-phenanthroline, from BDH, solution) was employed, the 8 washes of the cell suspension were followed by further 2 washes with dH₂O to completely remove any residual orthophenanthroline. Cell walls were digested and analysed by AAS as described in paragraph 2.6.1.

Figure 2.6 Yeast cell breakage by glass beads Yeast cells of lager strain B were collected during fermentation and broken by glass beads. Pictures represent untreated cells: control (a) and broken cell walls (b) visualised under bright field microscope (magnification 100x). Cell walls were then separated and washed several times with dH₂O and a solution of orthophenanthroline 0.01% as described in paragraph 2.8).



2.9 Atomic Force Microscopic analyses

Samples for AFM were prepared according to the method of Canetta *et al.* (2005) by spreading aliquots of 100µl of cell suspensions onto the surface of glass microscope slides. The cells were left to dry overnight at room temperature and visualised by using an AFM, a Nanoscope III (Veeco Instruments, Woodbury, NY). The AFM photographs were performed in air at room temperature (courtesy of Dr. Canetta).

2.10 Qualitative visualisation of zinc

Fluorescent images were taken using a Leica DMR microscope fitted with a Sony 3CCD colour video camera (model DXC-99OP). Cells were mounted on glass slides and placed under a 100x objective. Controls were visualised under bright field. Yeast cell zinc and vacuoles, previously stained with Fluo-Zn3 and Cell-tracker B (see paragraph 2.5.2) were visualised using, respectively, a green and a blue filter. Images were captured using the software Leica Qwin Standard V 2.6.

Flow cytometric analyses were carried out using a Cyflow Partec fitted with a solid-state laser output (532 nm) and a detector in the range 570-610 nm.

2.11 Statistical approaches

All experiments were performed in duplicate or in triplicate (for zinc limitation studies in chemostat). All analyses of samples collected from these experiments were carried out in either duplicate or triplicate depending upon the experimental conditions. This replication allowed for a mean and standard deviation of the individual data points to be calculated and showed that the data were reproducible.

Chapter 3

Zinc uptake by industrial yeast strains

3.1 Introduction

Zinc uptake by *S. cerevisiae* occurs in two phases: a metabolic energy-independent phase, based on the adsorption of zinc to the cell surface; and a metabolic energy-dependent phase, based on plasma membrane ATPase activity and generation of a transmembrane proton gradient, which mediates zinc uptake into the cell.

Glucose, as a source of energy, strongly stimulates zinc uptake. In yeast peptone dextrose medium (YPD) with various sugar concentrations (Hall, 2001), it was shown that industrial yeasts growing and fermenting in high sugar concentration (5%), accumulated higher levels of zinc compared to cells growing and respiring in low sugar concentration (0.1%). The explanation to this phenomenon was due to higher zinc requirements for alcohol dehydrogenase (ADH) synthesis and activity (Magonet, 1992). Since cells were found to accumulate zinc in the lag and early logarithmic phases of growth, it appeared that fermenting yeast cells acquired more zinc in order to stabilise the plasma membrane against possible damage provoked by increasing ethanol levels (Hall, 2001). Most likely, high levels of glucose provided enhanced energy to drive accumulation of intracellular zinc. The stimulatory effect of glucose on zinc uptake was inhibited by the following metabolic inhibitors: antimycin A (Mowll and Gadd, 1983), potassium cyanide, 2,4-dinitrophenol (DNP), DCCD, diethylstilboestrol (DES) and 2-deoxyglucose (White and Gadd, 1987). Although these compounds showed a clear metabolic dependent mechanism of zinc uptake, unfortunately they may interfere with various metabolic pathways or may have non specific effects. Therefore, they do not prove any specific mechanism implicated in zinc uptake.

In *Candida utilis*, zinc uptake was strongly impaired in starved cells by the presence of the protein synthesis inhibitor cycloheximide (Failla *et al.*, 1976). This demonstrated that proteins were involved in zinc uptake. More recently, in *S. cerevisiae*, discoveries were made of various protein transporters and their corresponding encoding genes, involved both in zinc uptake into the cell and in zinc compartmentalisation into various cell

organelles, including vacuoles (Zhao and Eide, 1996a, 1996b; Li and Kaplan, 1998a; Miyabe *et al.*, 2001; Li and Kaplan, 2001; MacDiarmid, *et al.*, 2000, 2002; Waters and Eide, 2002; Ellis *et al.*, 2004) (for details see sections 1.5.2 and 7.1).

Zinc uptake can be described by the Michaelis-Menten equation. Various dissociation constants have been found for yeasts, ranging from 10 to 1150 μmol zinc (Borst-Pauwels, 1981). This variation has been found even within the same yeast species and may be attributed to different techniques used to measure zinc or to the presence in the medium of buffers or complexing anions. More precisely for *S. cerevisiae*, two systems have been found: one high affinity system with $K_d = 10 \text{ nmol}$, active in zinc-limited cells (Zhao and Eide, 1996a) and one low affinity system with $K_d = 100 \text{ nmol}$, active in zinc-replete cells (Zhao and Eide, 1996b).

Growth medium pH may affect zinc uptake. In *Candida utilis*, zinc uptake rate decreased while pH increased from 4.8 to 8.2 (Failla *et al.*, 1976; Failla and Weinberg, 1977). This may have also depended on the reduced bio-availability of zinc which may form complexes with polyphosphates, carbonates and hydroxides at pH values over 6.8 (Ross, 1994) or the formation of zinc oxide, which depends on the level of oxidation of the medium (Pourbuaix, 1963). This phenomenon was also observed in *S. cerevisiae* cultures in the present study. For example, zinc depleted cells were found unable to grow in batch cultures when they were transferred from a medium at pH 4.7 to a medium at pH 6 (see chapter 7.1). With regard to temperature, Mowll and Gadd (1983) have observed a decrease in zinc accumulation in cells of *S. cerevisiae* when the temperature was reduced from 25°C to 4°C. The activation energy for zinc uptake was found to be 15 kcal (Ponta and Broda, 1970). The influence of temperature on zinc uptake was also found in the present study (see section Influence of low temperature on zinc uptake by a lager strain of *S. cerevisiae* in this chapter).

Uptake of heavy metals is usually accompanied by release of K^+ (Norris and Kelly, 1977; Lichko *et al.*, 1982; Gadd and Mowll, 1983). This phenomenon is not present in *S. cerevisiae* during zinc uptake, when zinc extra-cellular levels are at normal concentrations but it is usually observed when the concentrations become toxic and cells lose viability. In *Sporobolomyces roseus*, toxic levels of zinc and loss of viability were not accompanied with release of K^+ (Mowll and Gadd, 1983). Therefore in *S. cerevisiae*, toxic levels may

produce membrane damage and leakage of K^+ while in *Sporobolomyces roseus*, zinc toxicity may not produce membrane damage but most probably enzyme and protein denaturation within the cytoplasm.

In brewing, zinc concentration in malt wort ranges from 0.10 to 5 ppm (Rees and Stewart, 1998). A comprehensive study of worldwide malt worts has revealed that zinc levels vary from a minimal of 0.02 to 0.69 ppm with a mean of 0.16 ppm (Hage, 1983). Although generally present at high concentrations in brewing malts (20 ppm), during malt wort preparation, most of the zinc may be lost by chelation (see chapter 1.5.4). Therefore, in brewing, it is common practice to add zinc to yeast cells or to wort to avoid slow fermentations and improve fermentative performance. Zinc supplementation may be carried out during yeast propagation, fermentation, storage or acid washing (Taidi *et al.*, 2000), depending on individual brewery practice. Zinc is commonly added as zinc sulphate, because it is economically more convenient. In some cases commercial biological nutrients such as “*Servomyces*” may be used (see chapter 1.3.3). This is of particular convenience in Germany where beers are brewed following the rules of the German Purity Law “*Reinheitsgebot*”. Commercial nutrients based on various micronutrients, such as zinc, are also available for the wine making industry, e.g. “*Go-ferm*” from Lallemand (<http://www.vinquiry.com/pdf/GoFermJan2005.pdf>). Zinc uptake studies by industrial yeast strains have been carried out by Mochaba *et al.* (1996) in small scale 2 L fermenters and in 60 L fermenters, at 11°C, using malt wort with zinc levels below 0.75 ppm. They found an immediate zinc cellular increase in the first hours after inoculation, followed by zinc level fluctuations in the remaining days of fermentation. At end of fermentation, cells were repitched in fresh malt wort and they released part of the zinc previously accumulated which was reabsorbed again after the first hours of fermentation. These cells, containing relatively high levels of zinc and probably less vital, had very poor fermentative performance, with a longer lag phase and lower peak yeast counts. The authors warned against the use of cells with high zinc levels, because they were considered less vital. Probably the yeast used was particularly sensitive to low levels of zinc.

In grape wine must, zinc concentration ranges from 0.04 to 7.8 ppm and the average is 0.90 ppm (Cabanis and Flanzy, 1999). Average zinc concentration in beet molasses is 40 ppm while in sugar cane molasses is 13 ppm (Curtin, 1973) with optimal concentration for

ethanol production in the range 1-5 ppm (De Amorim, personal communication). In the literature, no studies have been reported on zinc uptake kinetics by wine yeast strains in wine must or by bioethanol yeast strains in molasses. Work conducted in this chapter aimed to provide some answers to these questions.

The influence of biological, physical and chemical parameters on zinc uptake kinetics in *S. cerevisiae* has not been completely investigated and many questions remain on the mechanisms involved in zinc uptake by industrial strains of *S. cerevisiae*.

3.2 Experimental

Experimental aims

The aim of this study was to investigate zinc uptake kinetics of various yeast strains in laboratory and industrial media (malt extract broth, malt wort and grape juice). The influence of physical and chemical parameters, such as the effect of temperature, different zinc salt supplements, supplementation times and antagonism with calcium was investigated in order to evaluate how different industrial conditions or practises may influence zinc uptake by yeast cells. Zinc uptake by yeast cells was also investigated after inhibition of protein synthesis and after killing cells by heat treatment. An attempt to use Na orthovanadate, an ATPase inhibitor, was done to study the role of this enzyme in zinc transport. It was hoped that these studies would give indications on how cellular metabolic status may influence zinc uptake by yeast strains in industrial growth medium.

Experimental approach

Five industrial yeast strains of *S. cerevisiae* have been employed in this study: three brewing strains, one wine and one whisky strain. Initial studies with *Schizosaccharomyces pombe* and *Kluyveromyces marxianus* were performed to evaluate if zinc uptake kinetics were merely species dependent. Experiments were carried out in shake flasks using malt extract broth, malt wort and grape juice as growth medium. Yeast growth was determined by haemocytometer. Zinc cellular content and supernatant zinc levels were analysed after nitric acid digestion by A.A.S. according to the protocol described in Materials and methods section (Chapter 2). Where indicated, magnesium and calcium levels were also determined. Intra-cellular and extra-cellular zinc concentrations were successfully verified after zinc balance calculations. Table 3.1 shows an example of zinc balance.

Table 3.1 Example of zinc balance in a brewing yeast strain growing in malt wort with normal and high levels of zinc. A lager yeast strain (lager B) was inoculated at 5×10^6 cells/ml in malt wort (OG 1060), and grown in shake flasks, at 25° C for 24 hours. Zinc levels were altered with the addition of calculated volumes of a 1000 ppm zinc acetate solution. Zn was measured both in cells and in supernatant and was expressed as fg/ml.

	Cell number cells/ml ($\times 10^6$)	Zn cell content (fg/cell)	Zn cell content ($\times 10^2$ ng/ml of cells)	Zn supernatant ($\times 10^2$ ng/ml)	Balance ($\times 10^2$ ng/ml)
<i>Zn normal</i> <i>(0.95 ppm)</i>					
0	74.8 *	12.26*	0.611	9.50	10.1
1	6.5	169.23	11	0	11
4	18.5	54.86	10.2	0	10.2
7	43.8	22.86	10	0	10
24	263	3.80	10	0	10
<i>Zn high</i> <i>(9 ppm)</i>					
0	74.8 *	12.26*	0.611	90	90.61
1	6.7	222.22	14.8	71.7	86.5
4	17.8	181.20	32.3	58.3	90.6
7	34.3	169.34	58.0	28.0	86
24	267	33.68	90	0	90

* the balance at time 0 was considered as follows: cell number and Zn cell content (fg/cell) were referred to the seed culture. 20 ml of seed culture (calculated to have an initial cell number of 5×10^6 in the 300 ml medium of experimental flasks) were harvested and cells washed after removal of the supernatant. Once added to experimental flasks, they increased the total zinc to 0.611×10^2 ng/ml.

Experimental procedure

Seed culture in shake flask at 25°C, 180 rpm, for 24 hours
(Zinc concentration variable depending on the medium)
↓
Inoculum of 5×10^6 cells/ml in shake flask at 25°C, 180 rpm, for 24 hours
↓
Sampling (at 0, 15, 30, 45 min, 1, 2, 3, 4, 5, 6, 7 and 24 hours)
for: cell number, viability, metal ion concentration in medium and metal ion cell content

Notes:

- In experiments with altered zinc levels and for zinc spikes, zinc was added to the medium prior to cell inoculum in the salt form of zinc acetate from a sterile stock solution (1000 ppm of zinc).

- In experiments with high zinc levels, zinc was added to the medium prior to cell inoculum in the salt form of zinc acetate, zinc sulphate and zinc chloride from sterile stock solutions (1000 ppm of zinc).
- In experiments with cells treated by heat at 65°C for 1 hour, cells from seed cultures were washed and resuspended in dH₂O prior to heat treatment.
- Inhibition of protein synthesis was performed by addition of cycloheximide (from Sigma) to a final concentration of 4 ppm in the growth medium.
- In experiments with altered calcium levels, calcium was added to the medium prior to medium sterilisation in the form of calcium sulphate. Malt wort used was pre-treated with Chelex (from BioRad), to reduced cation levels which were subsequently re-established to the desired levels by addition of calculated amounts of metal salts.

3.3 Results and discussion

Zinc uptake by various yeasts in Malt Extract Broth medium.

In malt extract broth, after 24 hours of growth, all zinc was completely removed from the medium by the yeast cells. Since this was observed in three different yeast species and in five different *S. cerevisiae* strains, this property was not strain dependent (Figs. 3.1a; 3.1.b). Intracellular zinc concentration depended on cell size. Small cells, such as *Kluyveromyces marxianus* (29 fL) exhibited high intracellular zinc concentrations (e.g. 0.082 g/L=1.25 mM). On the contrary, big cells, such as the ale strain of *S. cerevisiae* (263 fL) exhibited low levels of intracellular zinc concentration (e.g. 0.019 g/L=0.29 mM) (Fig. 3.1b). These expressions of mM zinc concentrations provide a rough (over) estimate since they do not account for bound zinc and assume zinc is freely soluble in the cytosol. Perhaps intracellular free zinc represents a fraction of these figures. For example intracellular free magnesium in yeast cells constitutes a very small fraction (1-10%) of total cellular magnesium (Walker, 1994). Although these data did not explain if zinc uptake was a phenomenon depending on the volume or the surface of the cell, they showed that cells had a high capacity to accumulate all zinc present in the medium. This may be related to the active metabolic status of the cells used as inoculum, since these were healthy and in late exponential phase.

*Zinc uptake by *S. cerevisiae* strains in malt wort.*

Zinc uptake by four strains of *S. cerevisiae* (winemaking, baking, brewing and distilling) in malt wort was analysed in the first 7 hours when cells were actively dividing (early exponential phase) and after 24 hours (early stationary phase). Fig. 3.2 shows zinc uptake versus yeast cell growth in the first 7 hours. Zinc accumulation was very rapid with cells reaching the highest zinc uptake after only 30 minutes following inoculation. During this period cell number did not increase. The same patterns were described in cultures of *Candida utilis* by Failla *et al.* (1976). In the remaining time of fermentation, zinc cellular content gradually decreased while cells were dividing. Zinc accumulated in the first hour of growth was all available in the medium which consequently became rapidly zinc depleted. Over the same period of time, Mg and Ca level fluctuations were not dramatic compared with zinc (Fig. 3.3). Magnesium levels decreased significantly only after 24 hours (data not shown) as found by Walker and Maynard (1997) while calcium residual

levels remained constant. The rapid uptake was only specific to zinc although zinc yeast cell requirements are usually much lower than for calcium and magnesium, the latter being considered a “bulk” cation (i.e. required at levels in excess of 100 ppm).

Malt wort is an industrial medium suited for growth of brewing or whisky yeast strains. Although this medium may not be optimal for growth of wine yeast strains, specifically selected for wine must fermentations, this yeast showed uptake patterns for metal ions similar to other industrial strains. While the type of medium used in these studies did not influence zinc uptake kinetics, sugar concentration may be a factor. Glucose is known to have a stimulatory effect on zinc cell uptake (Ponta and Broda, 1970; Hall, 2001). Most likely, actively growing yeast cells used as inoculum may have had sufficient intracellular energy (e.g. glycogen) to be utilised as driving energy necessary to accumulate intracellular zinc. In malt wort, this energy was provided by maltose (Kennedy *et al.*, 1997). This disaccharide is hydrolysed by the enzyme α -glucosidase into two glucose molecules (see paragraph 1.2.2) the metabolism of which can then stimulate zinc uptake.

The use of shake flasks keep cells homogeneously in suspension and exposes cells more efficiently to zinc ions present in the medium. In industry, although some agitation may be implemented, yeast cells are infrequently kept under conditions of homogenous culture. Therefore, in these experiments, agitation may have been an important factor contributing to high velocity of zinc uptake.

Figure 3.1. Zinc uptake by various yeasts in Malt Extract Broth medium. Two non-*Saccharomyces cerevisiae* species (*Shizosaccharomyces pombe* and *Kluyveromyces marxianus*) and five strains of *S. cerevisiae* including the wine strain B and the lager strain A were cultivated in shake flasks, in Malt Extract Broth (zinc at 0.30 ppm), at 25°C. Mean cell size (a) and mean zinc cellular concentration (b) were analysed after 24 hours of growth. Error bars are standard errors of the mean deviations, as for all graphs presented in this chapter.

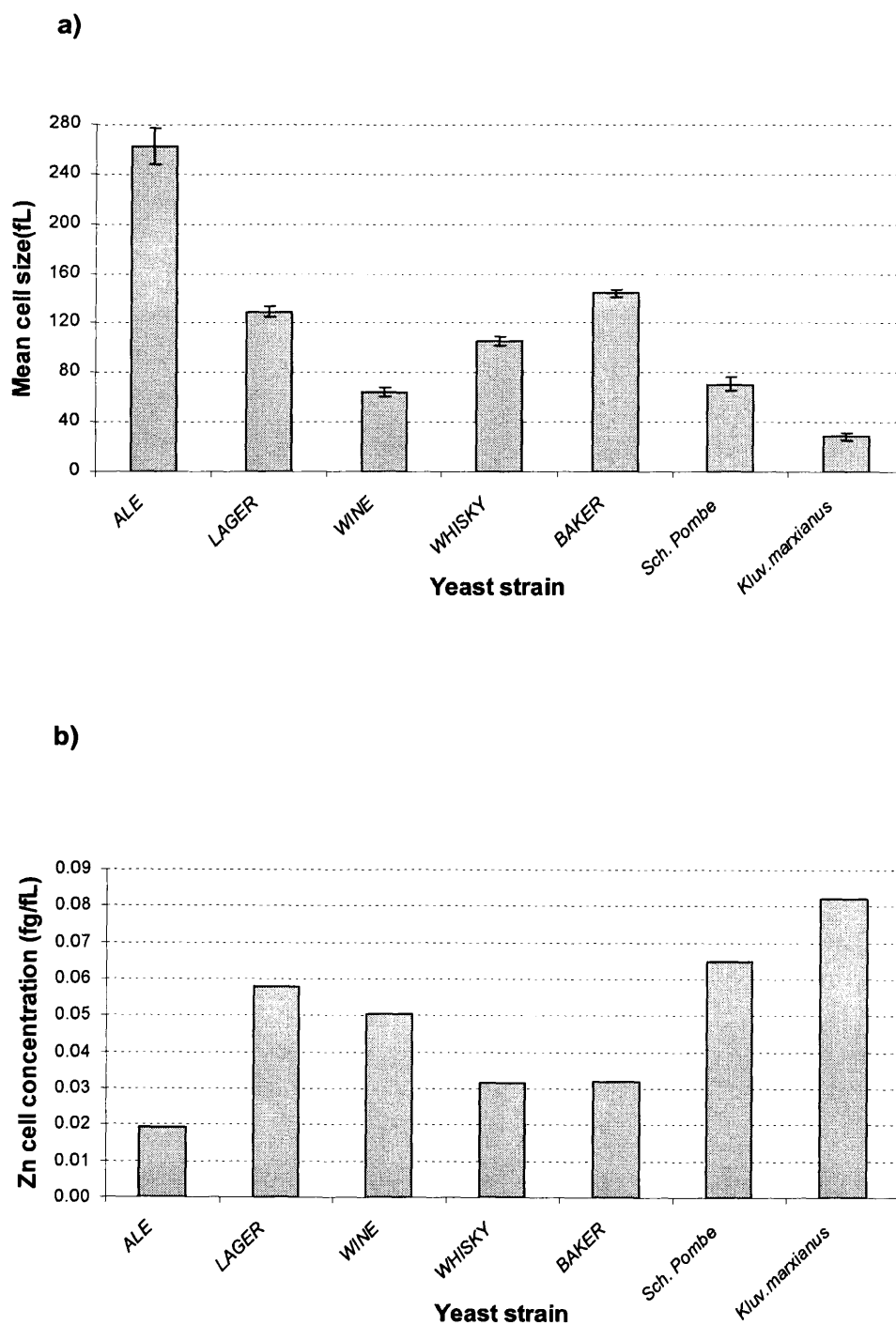


Figure 3.2 Zinc uptake by *S. cerevisiae* strains in malt wort. Four industrial strains of *Saccharomyces cerevisiae*, including the wine strain B and the lager strain A were cultivated in shake flasks in malt wort (zinc at 0.175 ppm), at 25°C for 24 hour. Cell growth versus zinc cell content was determined for lager (a), ale (b), whisky (c) and wine (d) yeast strains. Graphs show the first 7 hours of growth.

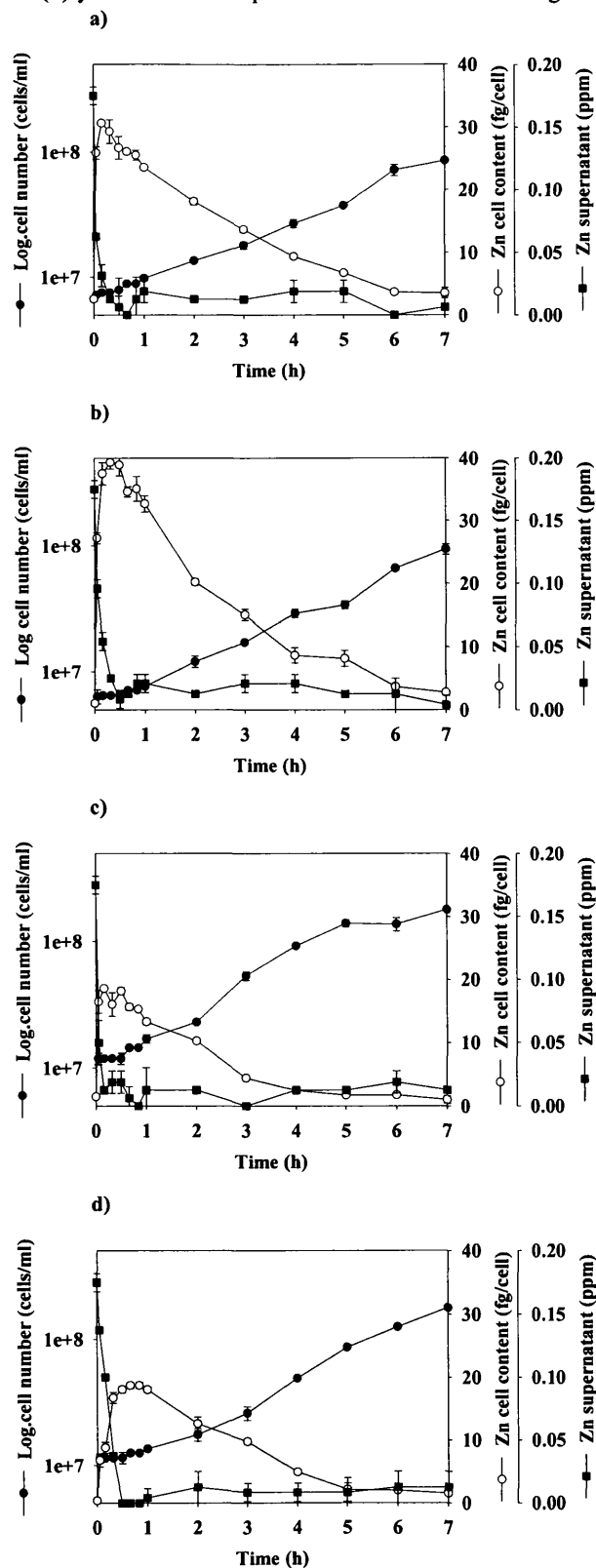
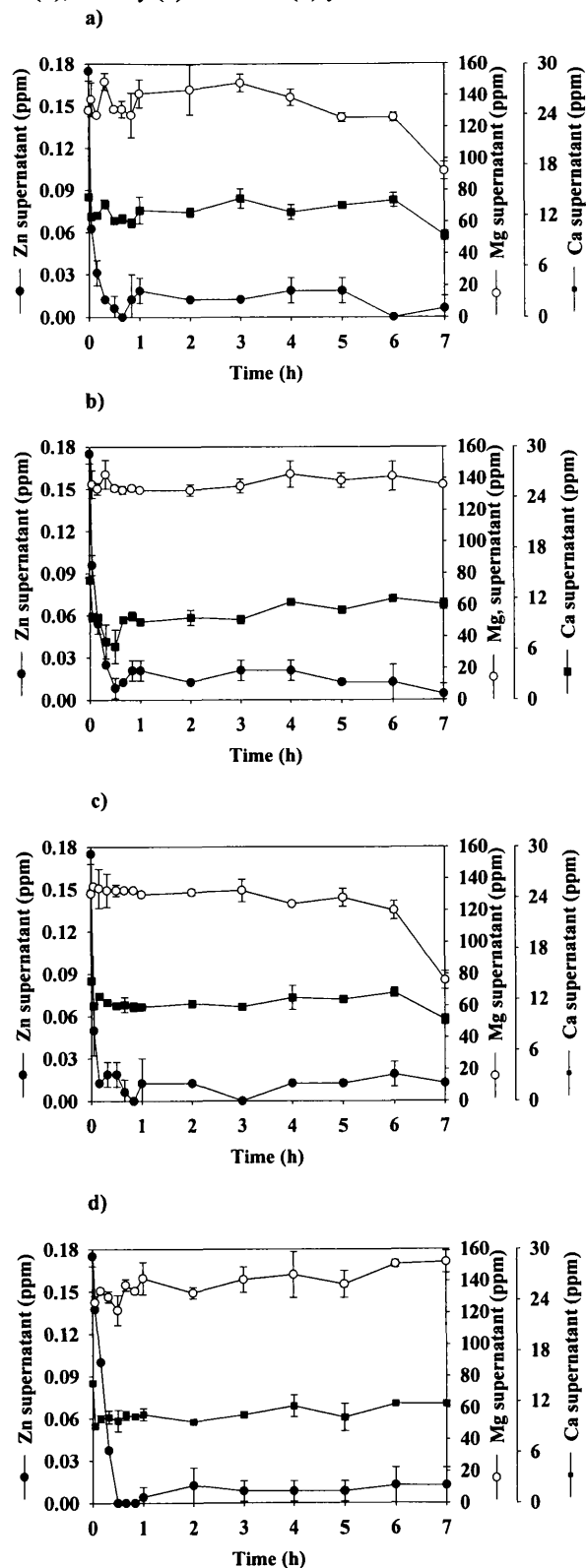


Figure 3.3 Zinc, calcium and magnesium uptake by *S. cerevisiae* strains in malt wort. Zn, Ca and Mg supernatant levels were analysed for 24 hours of growth. Graphs show the first 7 hours of growth of the following yeasts: lager (a), ale (b), whisky (c) and wine (d) yeast strain.



Influence of altered zinc concentrations on zinc uptake by a lager strain of S. cerevisiae

Alteration of zinc levels in malt wort media by addition of zinc acetate (0.35-3.55 ppm of zinc), resulted in complete zinc removal by the chosen lager brewing yeast strain (lager strain A). As described in the previous experiment, the uptake pattern was very fast. At lower zinc concentrations, zinc accumulation was very rapid, with cells reaching the highest zinc level after 30 minutes-1 hour (Fig. 3.4.a). At higher concentrations, a longer period of time (1-2 hours) was required to uptake all zinc from the medium (Figs. 3.4.c; 3.4.d). This time point corresponded with the highest cellular zinc level. After 5 hours, a zinc “spike” (Fig. 3.4.b) was performed to the medium having originally normal zinc levels (0.35 ppm). Zinc concentration was suddenly increased to 1.55 ppm. This spike resulted in an immediate zinc uptake response by yeast cells. After 5 hours, cells were still actively dividing and energy was available for metabolic functions and as driving energy for metal ion uptake. It would be interesting to evaluate zinc uptake capacity after zinc spike, when cells are in advanced stationary phase and with sugar availability consistently reduced. This may provide indications on how glucose availability influences zinc uptake in industrial media.

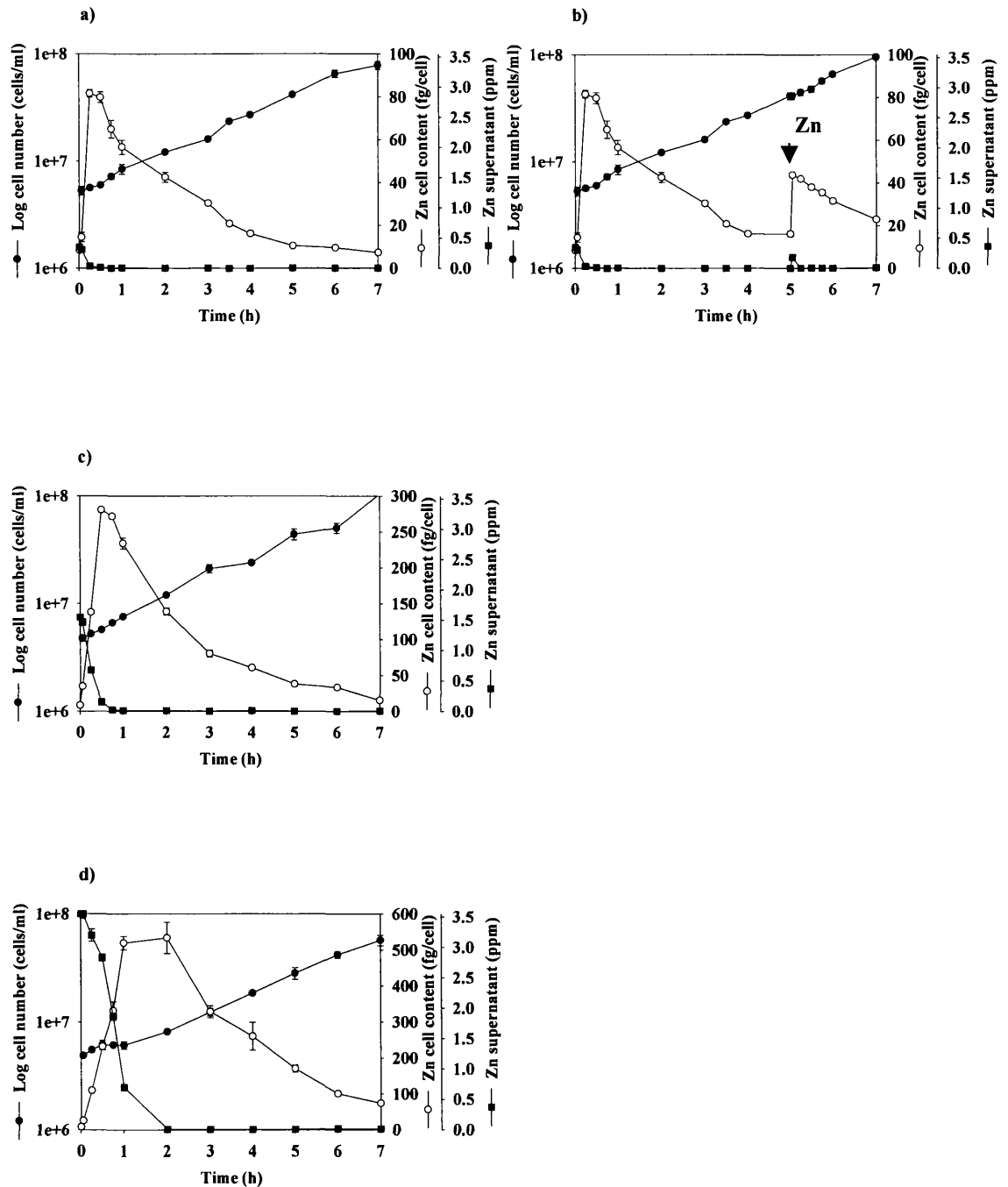
At all zinc concentrations under study, average zinc cellular contents decreased when cells started to actively divide. Growth curves, final cell numbers and culture viabilities (99%, data not shown) were not affected by various zinc concentrations. None of the zinc levels tested (0.35-3.55 ppm) was toxic to yeast cells. This may depend on the strain tolerance to elevated levels of zinc or to the presence of sufficient manganese levels (see also section 1.5.4) which were not analysed in this experiment. In this regard, extra-cellular manganese levels of 0.4 ppm were considered satisfactory for yeast to tolerate up to 65.5 ppm of zinc (Jones and Greenfield, 1984). Such manganese effects have never been reproduced in brewing fermentations (Dr. M. Walsh, personal communication).

Although these data need to be confirmed in bigger scale experiments, in industry, zinc supplementation calculations may be more meaningful at the beginning of fermentation, when sources of energy are abundantly available. On the contrary, zinc-depleted media may be detrimental since the “dilution effect” occurring during cell division will certainly concomitantly result in cellular zinc depletion. In brewing, yeast cells are often repitched for several cycles depending on the brewery. After one fermentation and prior to the following, yeasts may be stored in storage tanks where they are kept starved at low

temperature (4°C) and high cellular densities for variable periods of time. Where applied, cells may be washed with acids (e.g. phosphoric) in order to lower the pH and consequently to reduce numbers of contaminant bacteria (e.g. *Lactobacillus*). Some breweries add zinc during these steps to avoid the chelating effect of buffers present in malt wort. Zinc supplementation to storage tanks does not seem particularly effective considering that yeast cell slurries would have no sugar and therefore driving energy would not be available for zinc uptake. Also, low storage temperature would not be conducive to enhanced zinc uptake. Addition of zinc during acid washing was investigated by Taidi *et al.* (2000) who did not find any improvement in fermentation performance after zinc supplementation, concluding that yeasts did not accumulate sufficient zinc during this particular procedure. The same research proved that zinc addition to hot wort was also ineffective since zinc could be lost by chelation to the trub. The best time proposed for zinc supplementation was close to the time of pitching since zinc was considered more bioavailable. These conclusions were only based on the fermentative performance of yeast cells after zinc treatment while zinc cell contents were not investigated.

Although in this chapter, zinc cellular level was expressed as mean value, a better understanding of how zinc is distributed into cell populations would provide more useful information on how beneficial zinc supplementation may be. This topic will be dealt in more detail in chapter 4.

Figure 3.4 Influence of altered zinc concentrations on zinc uptake by a lager strain of *S. cerevisiae*. A lager brewing strain (lager strain A) of *S. cerevisiae* was cultivated in shake flasks in malt wort (OG 1060) at 25°C for 24 hour. Zinc concentrations were altered adding zinc acetate to the following levels: 1.55 ppm with spike (at arrow) after 5 hours (b), 1.55 ppm with zinc added prior to inoculum (c) and 3.55 ppm (d). Unaltered zinc level was also studied as control: 0.35 ppm (a), Graphs represent the first 7 hours of growth. Zinc cell content, zinc supernatant levels and cell number counts were performed throughout fermentation. The arrow in figure b represents the zinc spike.



Zinc uptake by whisky and wine strains of S. cerevisiae in industrial media.

Zinc uptake studies were conducted with whisky and wine (strain wine B) yeast strains in, respectively malt wort (without hop addition) and grape juice (Fig. 3.5.a; 3.5.b). Zinc uptake patterns were similar to those found in previous experiments with brewing yeast strains. Zinc was always completely and quickly up-taken by yeast cells, regardless of the type of media they were growing in. In grape juice, sugar concentration is usually higher than in malt wort and glucose and fructose are the main sources of carbohydrates available (Table 1.a). Therefore, the “driving energy” for zinc uptake is immediately available.

Influence of low temperature on zinc uptake by a lager strain of S. cerevisiae.

In the brewing industry, fermentations are usually carried out at lower temperatures than in wine making and whisky industry (see table 1.1). Consequently, lager yeast strain uptake studies were conducted both at 25°C and 8°C in order to evaluate zinc uptake patterns encountered in industrial brewing environmental conditions. In accordance with the work of Mowll and Gadd (1983), at lower temperatures, the accumulation of zinc by yeast cells was slower. Flocculent brewing yeast cells (lager strain B) growing at 8°C showed a higher peak in zinc uptake compared with cells growing at 25°C: 102 fg/cell compared with 85 fg/cell (Fig. 3.6.a; 3.6.c). However, complete zinc uptake was delayed after 6 hours at 8°C compared with only 45 minutes at 25°C. A non-flocculent brewing yeast strain (lager strain C) showed the same behaviour (Fig. 3.7.a; 3.7.c). The reduced capacity to uptake zinc at 8°C and the reduced growth rate of the yeast cells meant that the zinc content remained higher because it was not diluted by cell division. After 48 hours of fermentation, the final zinc cell content of the flocculent strain was almost the same at both the temperatures under study: 2.26 fg/cell at 25°C compared to 2.94 fg/cell at 8°C. Zinc was completely up-taken and final cell number was similar. The final cell content of the non-flocculent strain was higher at 8°C compared to 25°C because of the slow growth rate of the former: 3.42 fg/cell at 25°C compared to 18.50 fg/cell at 8°C. All zinc was removed from the medium. Magnesium and calcium levels in the supernatant were relatively unchanged at both the temperatures under study in both the flocculent and non-flocculent lager brewing strains (Fig. 3.6.b; 3.6.d; 3.7.b; 3.7.d). High zinc uptake was specific to zinc, even at lower temperatures.

Zinc accumulation at lower temperatures, was clearly impaired by the reduced metabolic activity. Temperature decreases reduced the activity of many enzymes and therefore also the metabolic mechanisms involved in transmembrane zinc uptake, facilitated by ATPase. Data obtained from this study are relevant to the brewing industry since zinc uptake may be consistently delayed during fermentation at low temperatures. Moreover, low temperatures in the storage tanks (4°C), together with the low sugar levels in yeast slurries, are not suitable to support an efficient zinc uptake when zinc supplementations are made during yeast cell storage. More complete studies on zinc uptake and fermentative performance in simulated small scale brewing fermenters and in 200 L brewing fermenters, at low temperatures, will be described in chapter 5.

Figure 3.5 Zinc uptake by whisky and wine strains of *S. cerevisiae* in industrial media. A whisky strain was cultivated in shake flasks in malt wort (zinc at 0.32 ppm), at 25°C for 24 hours (a). A wine strain (wine strain B) was cultivated in grape juice (zinc at 0.42 ppm), at 25°C for 24 hours (b). Figures represent cell growth, zinc cell content and zinc supernatant concentrations in the first 7 hours of growth.

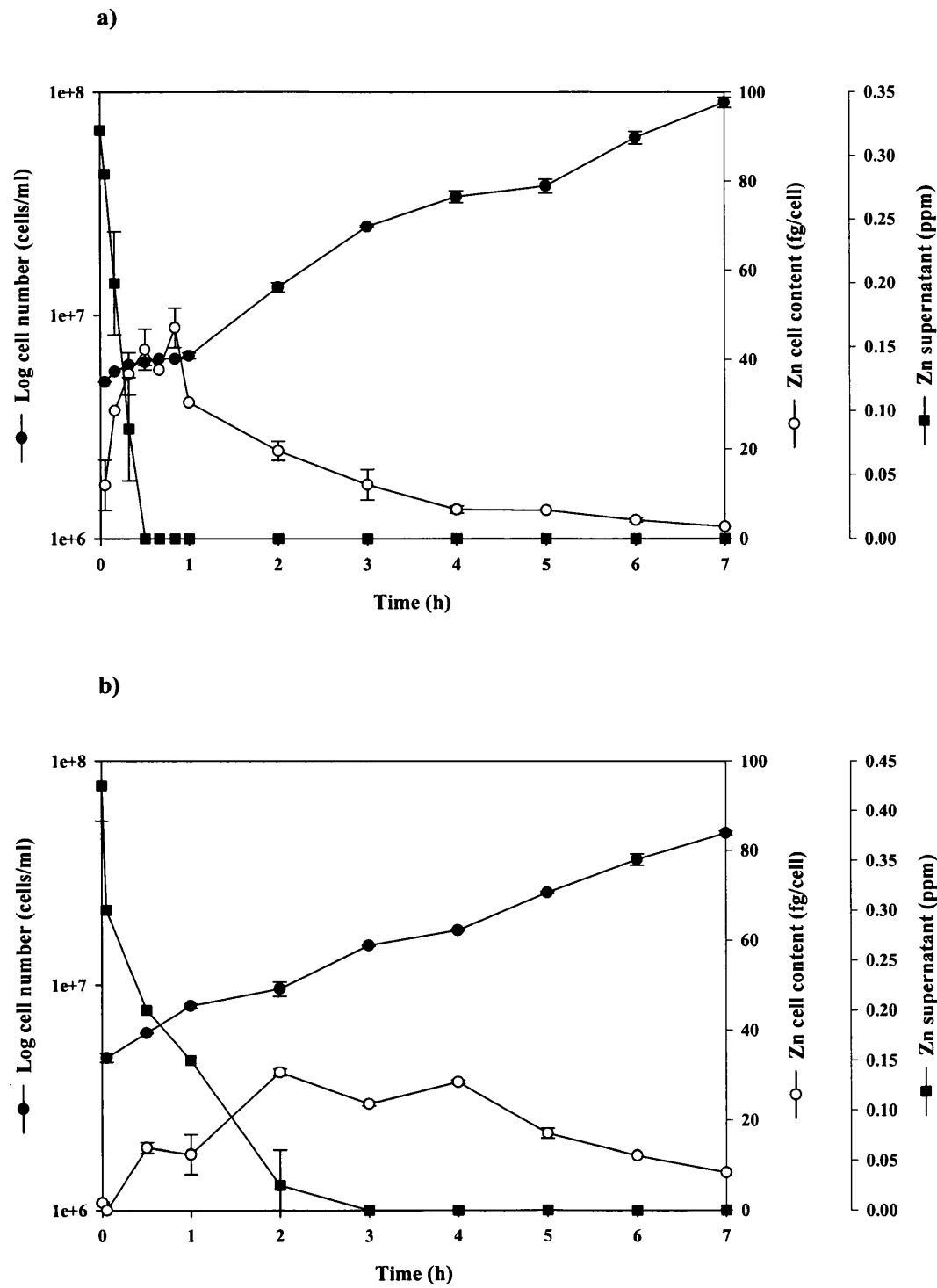


Figure 3.6 Influence of low temperature on zinc uptake by a flocculent lager strain of *S. cerevisiae*. A flocculent lager strain (lager strain B) was cultivated in shake flasks in malt wort (zinc at 0.54 ppm), at 25°C (a, b) and 8°C (c, d) for 48 hours. Figures represent cell growth versus zinc cell content and supernatant (a, c) and Zn, Mg and Ca in supernatant (b, d).

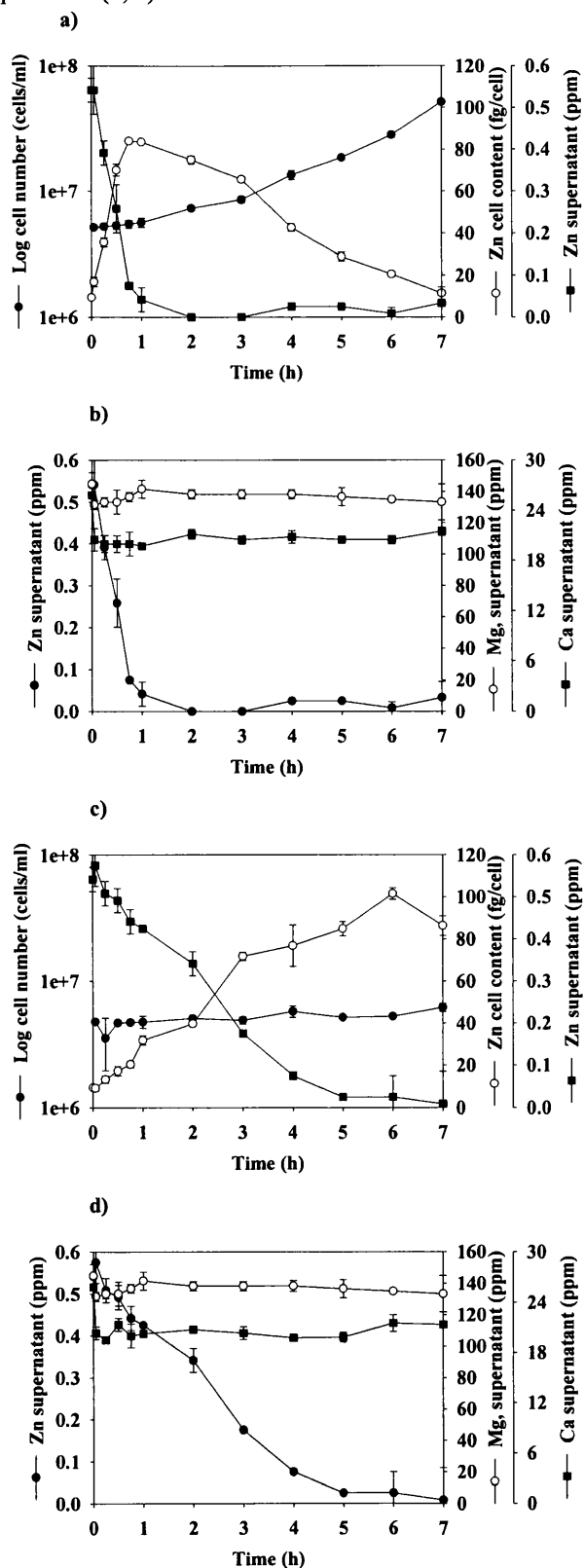
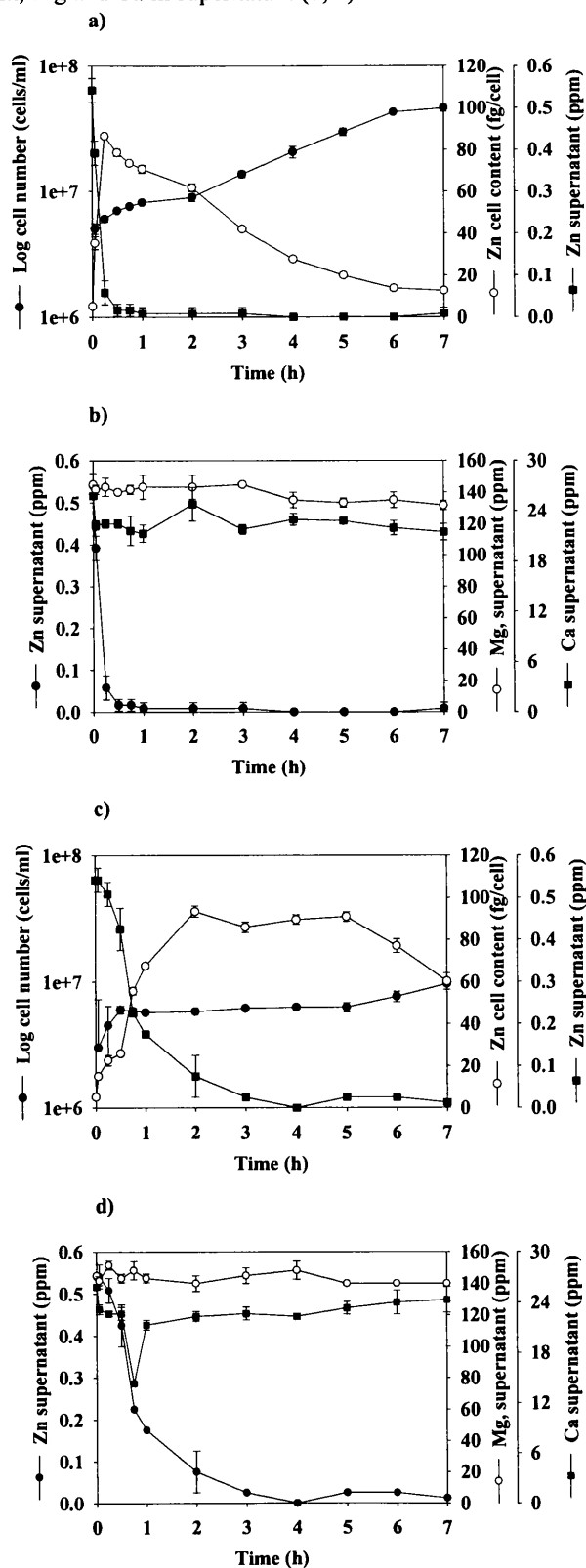


Figure 3.7 Influence of low temperature on zinc uptake by a non-flocculent lager strain of *S. cerevisiae*. A non-flocculent lager strain (lager strain C) was cultivated in shake flasks in malt wort (zinc 0.54 ppm), at 25°C (a, b) and 8°C (c, d) for 48 hours. Figures represent cell growth versus zinc cell content and supernatant (a, c) and Zn, Mg and Ca in supernatant (b, d).



Zinc uptake by dead cells of a lager strain of S. cerevisiae

Lager yeast cells of the lager strain B treated at 65°C for 1 hour died and therefore were not able to actively uptake zinc. More precisely, in the first hour, cells cultivated in Malt Extract Broth absorbed part of the zinc that was subsequently released back to the medium (Fig. 3.8.b). In normal conditions, all zinc would have been removed in the first hour (Fig. 3.8.a). Initial uptake by the dead cells was probably due to biosorption in that zinc formed bonds with the cell wall. This passive phenomenon was only short term and zinc was subsequently completely released back to the medium. The small amount of zinc uptake by biosorption showed that this phase was not the principal mechanism in healthy yeast cells. Therefore, the high zinc uptake shown by non heat treated cells demonstrated that the active metabolic phase was predominant compared to biosorption.

Heat preconditioned cells may have had their plasma membrane damaged by the high temperature. Heat stress may have increased fluidity to unstable levels resulting in breakdown of structure and function (Van Uden, 1984). Learmonth and Gratton (2002) reported that during heat stress, membrane fluidity irreversibly and progressively decreased. Most likely, membrane proteins were more vulnerable to oxygen-derived free radicals, which partition into membranes.

As a consequence, the maintenance of high viability yeast for brewing and distilling will ensure maximum zinc uptake during fermentation.

Zinc uptake by protein synthesis inhibited cells of S. cerevisiae

Failla (1976) demonstrated that in *Candida utilis*, the synthesis of new proteins played a key role in zinc uptake when cells had been starved. The protein synthesis inhibitor cycloheximide strongly reduced zinc uptake of these cells. In this Thesis, preliminary experiments showed that a concentration of 4 ppm of cycloheximide stopped cell growth of the lager strain B of *S. cerevisiae*, without affecting cell viability. This concentration was employed in experiments on zinc uptake. The lager strain B was found able to uptake all zinc from the medium (Fig. 3.9.b) at the same rate as non-treated cells (Fig. 3.9.a). Zinc was completely removed from the medium in the first hour of growth and the mean cell content remained very high in treated cells. In fact, these cells were not able to grow and to share zinc during cell division. This suggests that the *de novo* protein synthesis was not a crucial factor in zinc uptake. Pre-constituted proteins may have transported zinc ions

into the cells. It is possible that these proteins were not constituted in starved cells of *Candida utilis* employed in the study of Failla (1976).

Unfortunately, attempts to inhibit the membrane ATPase using Na orthovanadate were not successful since this compound changed form together with pH. At pH 4.5 this compound was toxic to yeast cells and at pH 5.5 yeast cells tended to form filaments. At pH 10 the inhibitory effect of Na orthovanadate worked best but yeast cells were unable to grow. The use of other known ATPase inhibitors (e.g. Dio-9, miconazole, suloctidil, N,N'-dicyclohexy-carbodiimide, triphenyltin and diethylstilbestrol) have been discouraged by Borst-Pauwels *et al.* (1983) for studies on the role of ATPase in yeast membrane transport processes because they drastically change cell membrane permeability, producing loss of K^+ and altering nutrient uptake.

Figure 3.8 Zinc uptake by dead cells of a lager strain of *S. cerevisiae*. Yeast cells of the lager strain B were cultivated in shake flasks in Malt Extract Broth (zinc at 0.25 ppm), at 25°C for 24 hours. Figures represent zinc cell content, zinc residual levels and cell growth in normal conditions of viability (a) and cells previously killed by heat treatment, at 60°C for 1 hour (b).

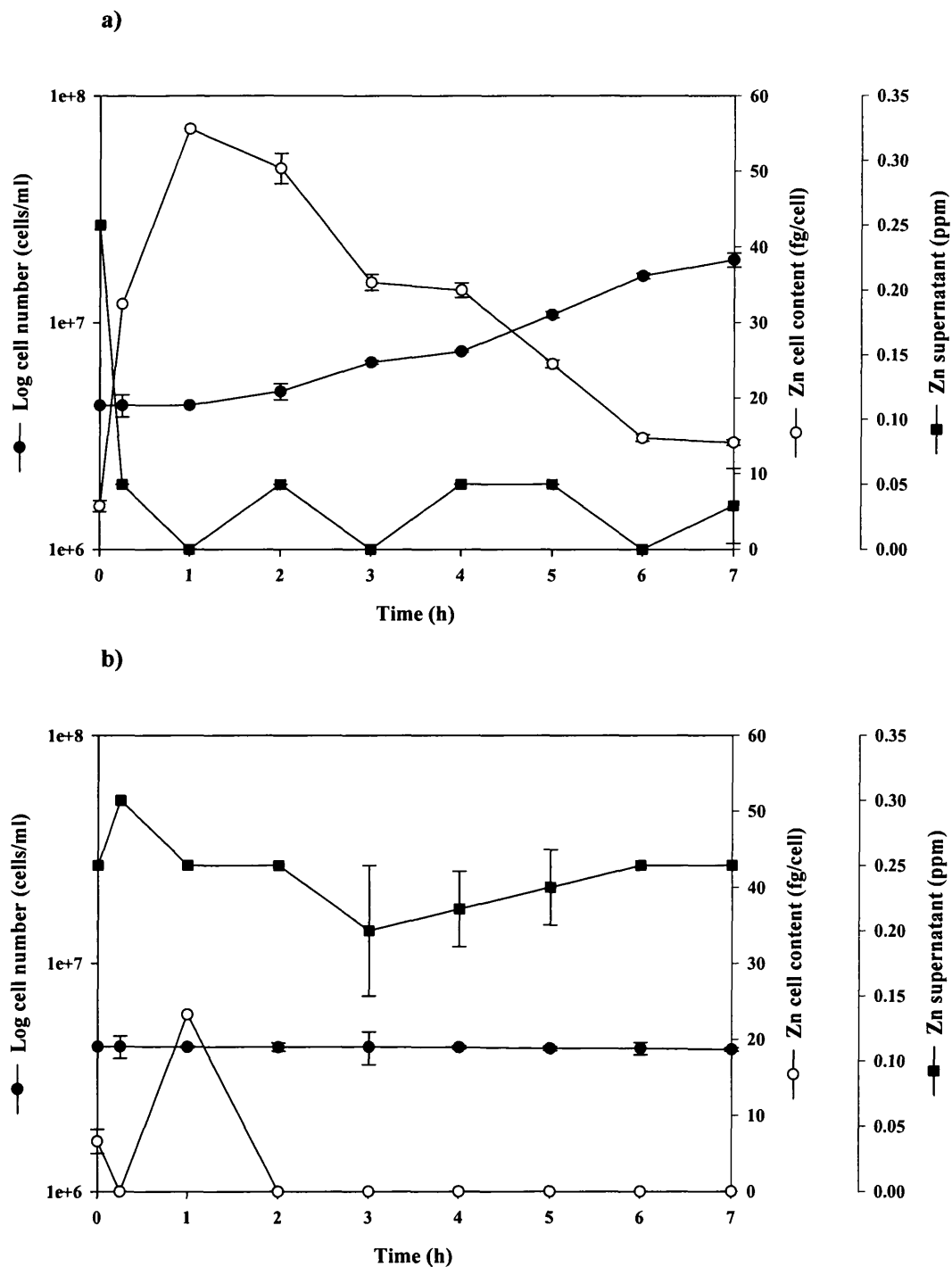
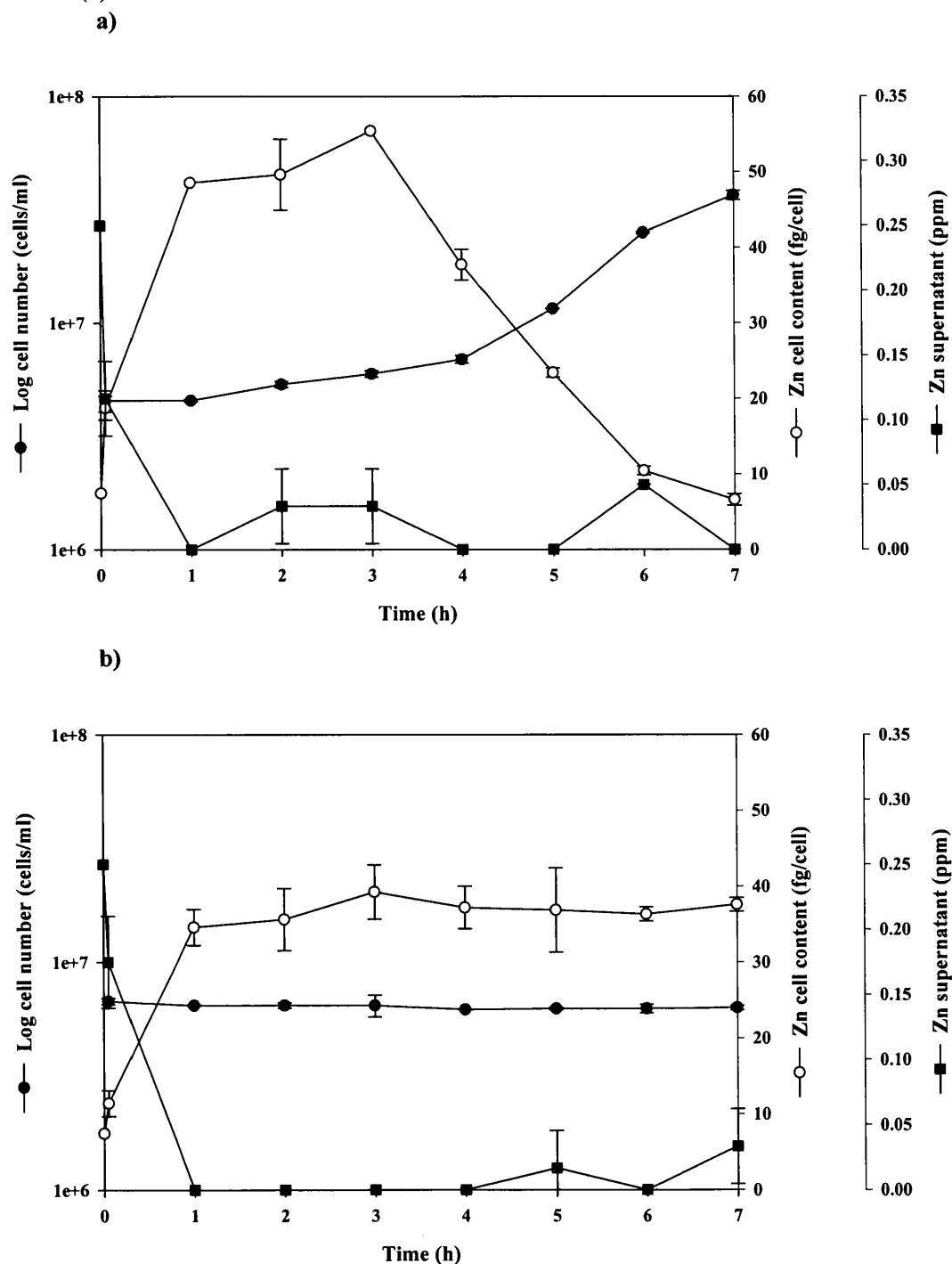


Figure 3.9 Zinc uptake by protein synthesis inhibited cells of *S. cerevisiae*. Yeast cells of the lager strain B were cultivated in shake flasks in Malt Extract Broth (zinc at 0.25 ppm), at 25°C for 24 hours. Figures represent cell growth under normal conditions of viability (a) and cells growing in medium with 4 ppm of cycloheximide (b).



Influence of calcium on zinc uptake by a lager strain of S. cerevisiae.

Zinc uptake may be influenced by the presence of other metals. In brewing, calcium may be the major antagonist among divalent cations present in malt wort. During brewing fermentations, it is common practice to add calcium based salts to adjust pH of the wort or to speed up yeast flocculation in order to simplify beer clarification process. Walker (1999b) warned against the use of calcium in brewing since it is an antagonist to magnesium-dependent functions. Moreover, water used for mashing may have high concentrations of calcium. As a result high calcium levels may be commonly encountered in brewing.

To evaluate the influence of calcium on zinc uptake, zinc wort levels were kept low (zinc at 0.15 ppm) and calcium levels were adjusted in the range 16-76 ppm (Fig. 3.10) by addition of calcium sulphate. This range was chosen as it was representative of calcium levels found in different malt worts (Dr. M. Walsh, personal communication). At various concentrations of calcium studied, zinc uptake patterns by the lager strain B were unchanged. Zinc was removed from the medium after only 30 minutes when cells reached the highest zinc cell content (Fig. 3.10). Although calcium levels were in the ppm range, cells tended to keep very low intracellular contents of calcium. At the beginning of fermentation, intracellular calcium levels fluctuated (Fig. 3.11). At end of fermentation, the final intracellular content of calcium was even lower than zinc, although zinc concentration in the medium was in the sub-ppm range (0.15 ppm). Cellular capacity to keep low calcium levels was already observed by Walker (1999b) who prescribed to calcium a secondary importance compared to magnesium in yeast physiology. It may be concluded that calcium does not influence zinc uptake, even at relatively very high concentrations found in brewers' wort.

Influence of various zinc salts on zinc uptake by a lager yeast strain of S. cerevisiae

Zinc uptake by yeast cells of the lager strain B was also studied under very high zinc concentrations. Zinc wort levels were brought up to 22 ppm by adding three different salts (zinc acetate, zinc sulphate and zinc chloride). Choice of such a high concentration was made to better appreciate zinc uptake throughout time of fermentation. Positive influences of magnesium uptake by yeast has already been shown by Smith (2001) when magnesium acetate was provided. This anion consistently increased the content of magnesium

compared to magnesium sulphate after 24 hours. Therefore, the correct choice of a salt to be used for zinc supplementation may be of importance in order to stimulate fast and efficient zinc uptake. This choice may be also influenced by the cost of the salt in question, particularly for industrial brewing operations.

Yeast cell growth rate and final cell number were not influenced by the type of zinc salt and they were the same as in the control culture (zinc at 0.37 ppm) $\sim 2.40 \times 10^8$ cells/ml (Fig.3.12). Cell viability was not influenced by high zinc levels and remained the same as the control with zinc at 0.37 ppm (99%). Therefore, high zinc levels were not toxic to yeast cells. Zinc toxicity in yeast cells has been reported to cause inhibition of H^+ efflux and K^+ uptake, reduction of viability, K^+ efflux and changes in the kinetics of Zn^{2+} uptake (White and Gadd, 1987). Formation of bonds with inappropriate sites in proteins or cofactors, degradative enzymes activation, enzyme denaturation, autolysis induction by activating proteolysis, inhibition of transport systems for essential ions and nutrients, plasma membrane fluidity alteration and leakage of K^+ (Rees and Stewart, 1998) are all the possible effects of zinc toxicity.

It is conceivable that in this experiment manganese levels in malt wort were sufficient to counteract any toxicity from zinc. Helin and Slaughter (1977b) observed that only 0.6 ppm of zinc were toxic if manganese levels were below 0.01 ppm. Although cell viability remained high, cell vitality may have been reduced and therefore the fermentative performance or the capacity to resist stress may have been affected. Since the aim of this chapter was to evaluate zinc uptake kinetics during fermentation, other physiological parameters were not considered here.

Maximum zinc cell uptake occurred after 2 hours of fermentation irrespective of the zinc salt studied. This time point did not correspond with the complete removal of zinc from the medium. At the end of fermentation, final zinc cell content was higher in cells pretreated with zinc acetate and zinc ions were still present in the medium: 5.80 ppm in the case of zinc acetate, 6.60 ppm with zinc chloride and 8.40 ppm with zinc sulphate, respectively, corresponding to 25.8%, 29.5% and 38.2% of the initial zinc. This suggests that the acetate anion slightly altered the uptake of zinc. It may be assumed that acetate may pass through the cell membrane by diffusion (Stratford and Anslow, 1998), reducing the intracellular pH, influencing cell membrane permeability and having synergistic effect on the H^+ -pumping ATPase. In addition, acetate can be used as an alternative carbon

source where it is oxidized in the glyoxylate cycle, which bypasses two carboxylation steps in the TCA cycle (Walker, 1998). Such metabolism may result in the co-translocation of acetate and zinc when conditions favouring the glyoxylate cycle prevail (sugar limitation, oxidative growth).

During the initial stages of the fermentation, part of the intracellular zinc was released back into the medium (Fig. 3.12) and taken up subsequently. Yeast cells may have developed systems to actively extrude zinc from cells in order to keep intracellular levels sub toxic. Probably during active cell division, zinc intracellular requirements may have been lower than in the following stages of fermentation. At the end of fermentation, reduced glucose availability and consequently decreased availability of energy sources may have also contributed to curtail zinc uptake. In this regard, an investigation of zinc uptake at high zinc levels may be interesting under glucose fed-batch cultivation conditions. The available energy may support complete zinc uptake.

Figure 3.10 Influence of calcium on zinc uptake by a lager strain of *S. cerevisiae*. The lager strain B was cultivated in shake flasks in malt wort (zinc at 0.15 ppm) with various calcium concentrations: 16 ppm (a); 40 ppm (b); 60 ppm (c); 76 ppm (d), at 25°C for 24 hours. Figures show: cell growth, zinc cell content, zinc in supernatant over the first 7 hours of growth.

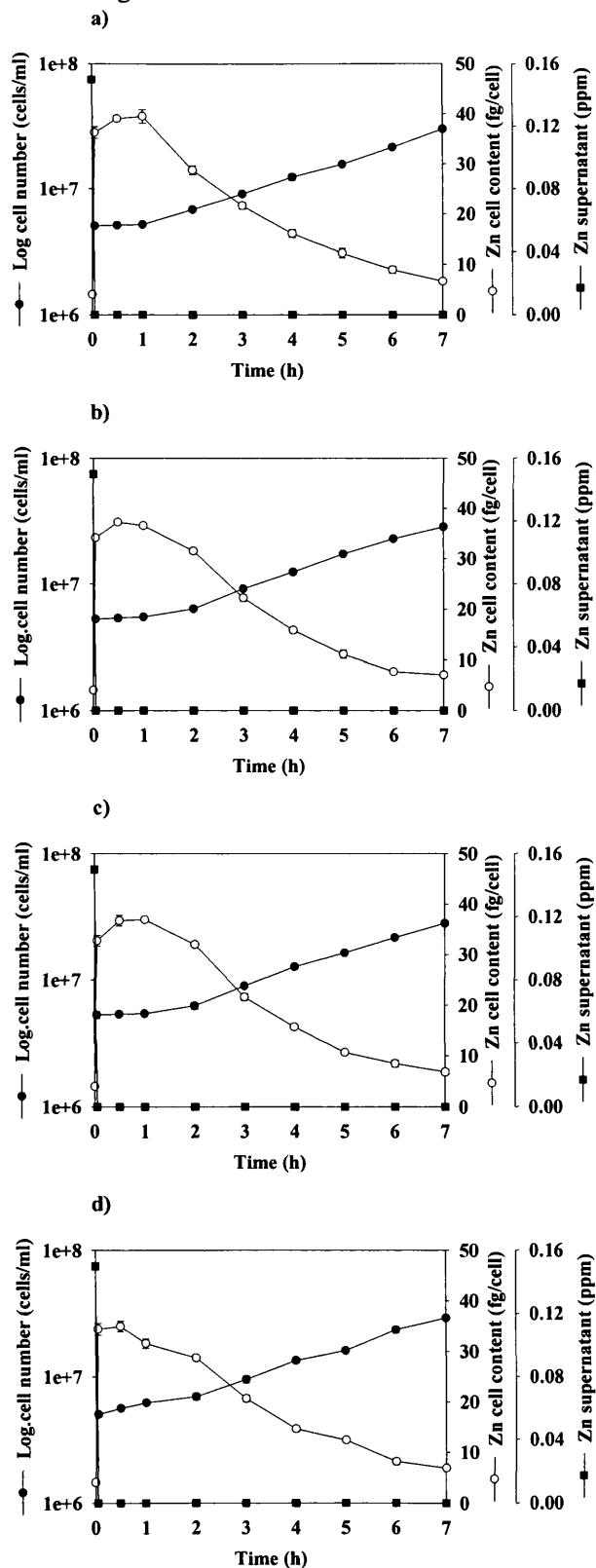


Figure 3.11 Influence of calcium on calcium uptake by a lager strain of *S. cerevisiae*. Calcium cell content (of the lager strain B) and calcium in supernatant were analysed over the first 7 hours of growth at any altered level of calcium: 16 ppm (a); 40 ppm (b); 60 ppm (c); 76 ppm (d).

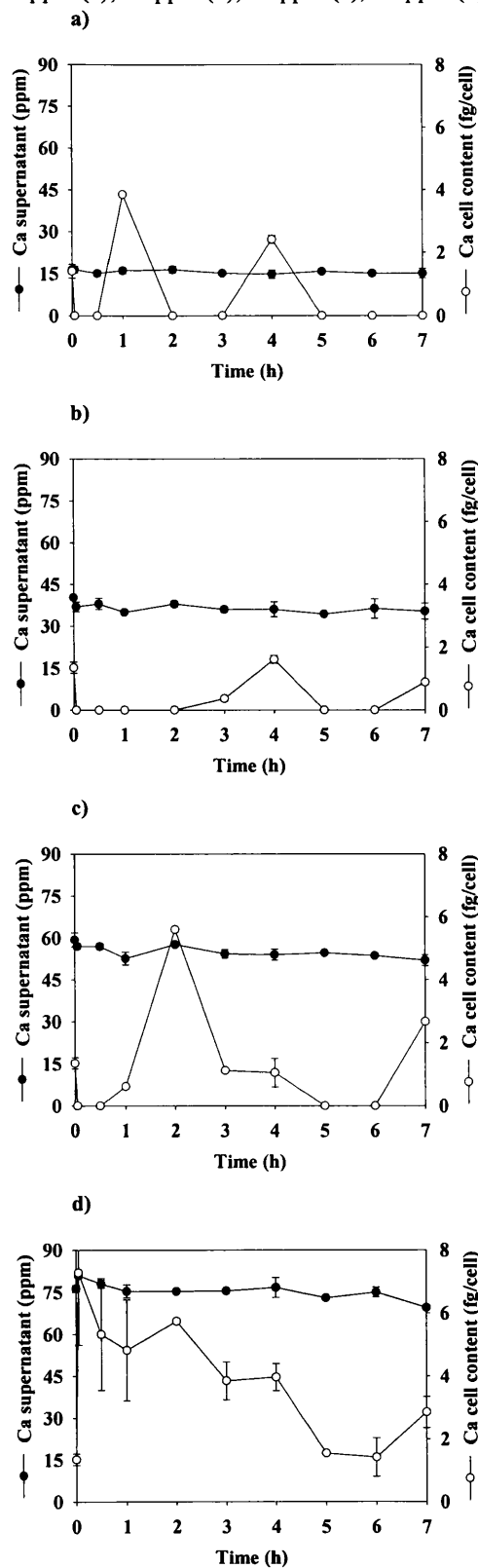
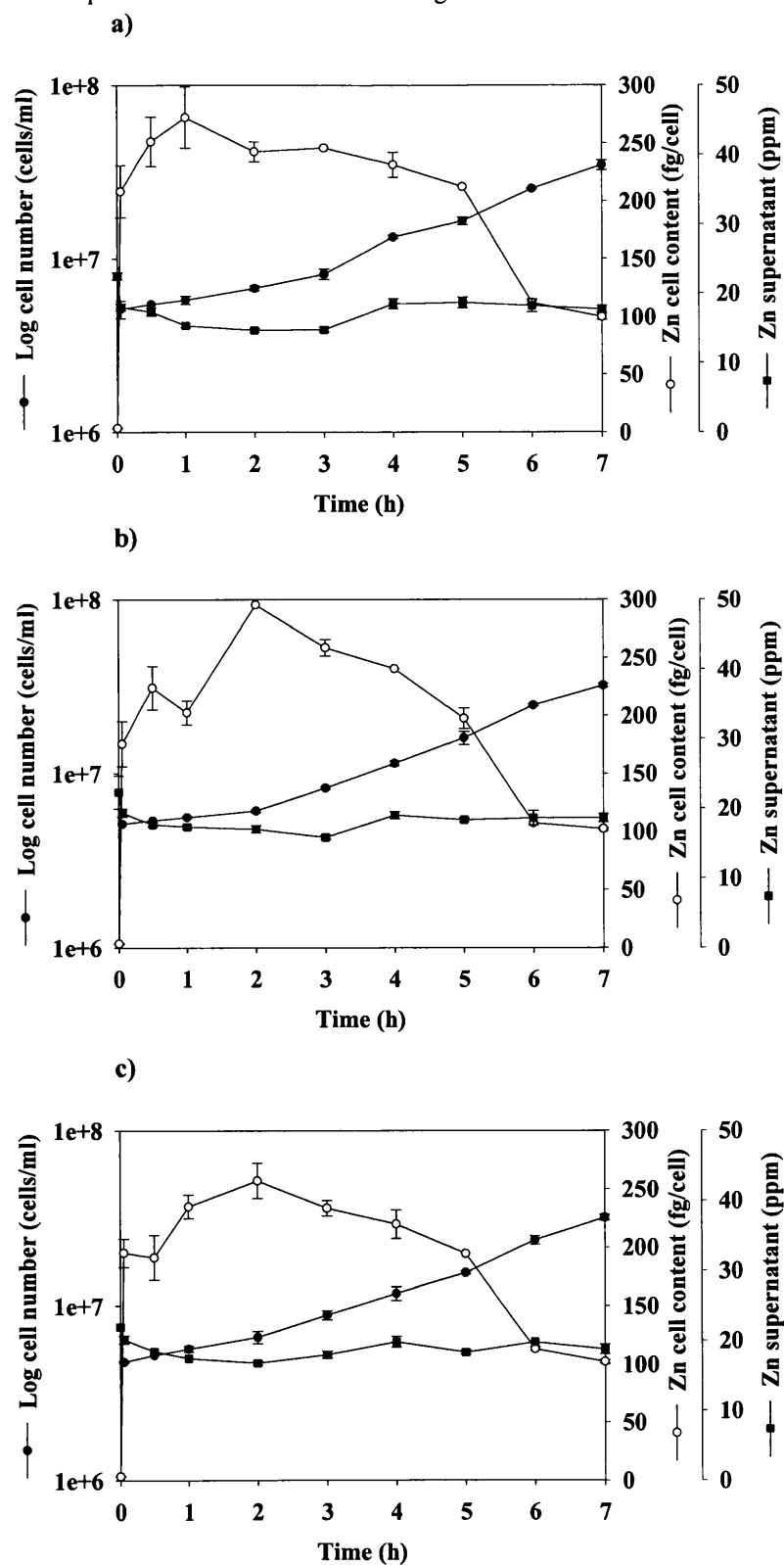


Figure. 3.12 Influence of various zinc salts on zinc uptake by a lager strain of *S. cerevisiae*. The lager strain B was cultivated in shake flasks in malt wort, at 25°C for 24 hours. Zinc level was altered to 22 ppm by addition of zinc acetate (a), zinc chloride (b) and zinc sulphate (c). Figures show: cell growth, zinc cell content and zinc in supernatant over the first 7 hours of growth.



3.4 Summary

The results of the research presented in this chapter showed that:

- Zinc uptake by industrial yeast strains was generally very fast and the element entirely disappeared from the medium. Zinc supplementations in the first hours of growth also resulted in quick uptake by the yeast cells.
- Zinc uptake was influenced by temperature (e.g. reduced at lower temperatures).
- Zinc uptake was metabolism dependent. Dead cells failed to take up zinc while uptake in protein synthesis inhibited cells was unaltered.
- Calcium concentrations (range 16-76 ppm) did not significantly influence zinc uptake.
- Various zinc salts (22 ppm of zinc) added did not significantly influence zinc uptake.
- Zinc uptake was metabolism-dependent. During brewing processes, it is suggested to add zinc at time of pitching, when optimal sugar levels are available as sources of driving energy. For the same reason it is discouraged to add zinc at low temperatures ($\sim 0^{\circ}\text{C}$) and when sugar sources are minimal (e.g. during acid-washing or yeast storage).

Chapter 4

Cellular localisation and distribution of zinc by a brewing yeast strain

4.1 Introduction

Zinc movement within the yeast cell occurs through various barriers. The first obstacle is represented by the cell wall. The cell wall constitutes 15-25% of the dry weight of the cell and it is essentially composed of a highly dynamic structure that is responsible for protecting the cell from rapid changes in external osmotic potential (Levin, 2005).

The cell wall is mainly composed of polysaccharides (80-90%) while proteins and lipids are present in lower amounts. Among polysaccharides, glucans (between 30 and 60%, depending on culture conditions) and mannans (between 25 and 50%) are the most important, with chitin present only in small percentage (Fleet, 1991). Specific binding regions for heavy metals are present on the cell wall which may change depending on the yeast strain or the media in use (Engl and Kunz, 1995). The zinc specific binding in the cell wall is the sulphhydryl group of cysteine located in the mannoprotein fraction (Brady and Duncan, 1994; Mochaba *et al.*, 1996). Zinc forms bonds with this group and in this Thesis it has already been demonstrated how these bindings may be loose (see chapter 3, paragraph on uptake of zinc by dead cells killed by heat treatment).

An energy dependent metabolism acting during zinc uptake has been reported in several studies. For example, White and Gadd (1987) determined the proportion of compartmented zinc within the cell: 56% in the soluble vacuolar fraction, 39% bound to insoluble components and only 5% was found in the cytosol. An ATP-dependent zinc uptake system whose properties were consistent with a $\text{Zn}^{2+}/\text{H}^{+}$ antiport was localised in isolated yeast vacuoles (White and Gadd, 1987) (see also Tab. 1.2). Lichko *et al.* (1982) have previously reported that intracellular K^{+} is concentrated in the vacuoles and that yeast cells may lose up to 30% of vacuolar K^{+} as they accumulate divalent cations. As previously mentioned, this phenomenon was only observed in *S. cerevisiae* when extra-cellular zinc levels were toxic. More recently, three specific zinc transporters have been localised in the vacuole: Zrc1 (Miyabe *et al.*, 2001), Cot1 (Li and Kaplan, 1998a) and Zrt3 (MacDiarmid, *et al.*, 2000, 2002) (for details see paragraph 1.5.3). MacDiarmid *et al.* (2003) were also the first

to visualise free zinc ions into the vacuole using the specific zinc fluorescent probe Fura Zin-1. Devirgiliis *et al.* (2004) used a different zinc specific dye (Zinquin) and were able to observe fluorescence in small vesicular compartments placed at the periphery of the cytoplasm (Fig. 1.3, Chapter 1)

In brewing experiments conducted in 60 L fermenters, Mochaba *et al.*, (1996) have analysed yeast cellular zinc localisation throughout fermentation, finding high zinc concentrations in the mannoprotein and in the intracellular fractions. Further, at the onset of the fermentation, zinc ions were translocated to the outer surface presumably to aid in maltotriose uptake.

4.2 Experimental

Experimental aims

The aim of this study was to determine the proportion between zinc accumulated on the cell wall and into the cell during various stages of yeast cell growth. Free zinc ions were also visualised by microscopic imaging after staining with zinc-specific fluorescent dyes. Information about intracellular zinc localisation is important to understand uptake and storage mechanisms occurring during cell growth.

The proportion of zinc shared between mothers and daughters during cell division was also investigated to determine how zinc was distributed within yeast cell population. Since zinc is uptaken within the first hour from industrial media and the mean zinc cell content gradually decreases with time (see chapter 3), this study will give a valuable picture on zinc cellular distribution. This information has a practical relevance to industrial processes when yeast biomass is re-used for several fermentations as in brewing and bioethanol industries.

Experimental approach

A lager yeast strain (lager strain B) was employed in this study. Growth was carried out in shake flasks, in malt wort for studies on total/cell wall zinc content and zinc population distribution and in EMM3 minimal medium for studies on free zinc intracellular localisation. The latter medium was chosen to reduce auto-fluorescence background interferences, normally encountered in complex media (Fig.4.2).

Experimental procedure

Zinc in yeast cell walls and protoplasts

Seed culture in shake flask, in malt wort at 25°C, 180 rpm, for 24 hours



Inoculum of 5×10^6 cells/ml in shake flask at 25°C, 180 rpm, for 24 hours
(zinc concentration variable altered by calculated additions of zinc acetate)



Sampling (at 0, 1, 4, 7 and 24 hours in experiment with dH₂O and at 0, 1, 3, 5 and 24 hours
in experiments with orthopenantrolin 0.01%)
for: cell number, zinc total cell content and zinc cell wall content.
Details on cell wall breakage and wash are given in chapter 2 (Material and methods).

Intracellular zinc localisation in yeast by fluorescence microscopy

Seed culture in shake flask, in EMM3 minimal medium (Zn at 0.5 ppm) ,
at 25°C, 180 rpm, for 24 hours



Inoculum of 5×10^6 cells/ml in shake flask, in EMM3 minimal medium (Zn at 10 ppm), at
25°C, 180 rpm, for 1 hour



Staining with Fluo-Zn3 fluorescent probe 10 µM and Cell-Tracker B 10 µM
Details on cell wall staining are given in chapter 2 (Material and methods)



Visualisation by fluorescent microscopy

Zinc distribution in yeast population

Seed culture in shake flask, in malt wort (Zn at 0.25 ppm) at 25°C, 180 rpm, for 24 hours



Inoculum of 5×10^6 cells/ml in shake flask in malt wort (Zn at 24 ppm) at 25°C, 180 rpm, for 16 hours and harvested in late exponential phase.



Cells washed once in dH₂O and layered onto the surface of 40 ml lactose gradient solution (from 30% to 15%)



Centrifugation of cell suspension in lactose gradient solution for 10 min, 1060 rpm, 20°C.



Sampling of cells at various levels by syringe (about 4 ml) to collect cells of different sizes



Analyses of zinc cell content

Notes:

- for flow-cytometric analysis, the medium used was malt extract broth with zinc concentration at 0.25 ppm. Cells were harvested after 14 hours of growth. Because of the low levels of zinc, undetectable by AAS due to its limit of detection, total zinc cell content was not analysed. Analyses were instead conducted by Flow Cytometry to determine levels of fluorescence emitted at various zinc levels. Cells of different size were diluted to the same cell density, stained by Rhod-Zn1, a specific zinc probe (see procedure in Materials and methods) and analysed using a Partec Cyflow flow cytometer.

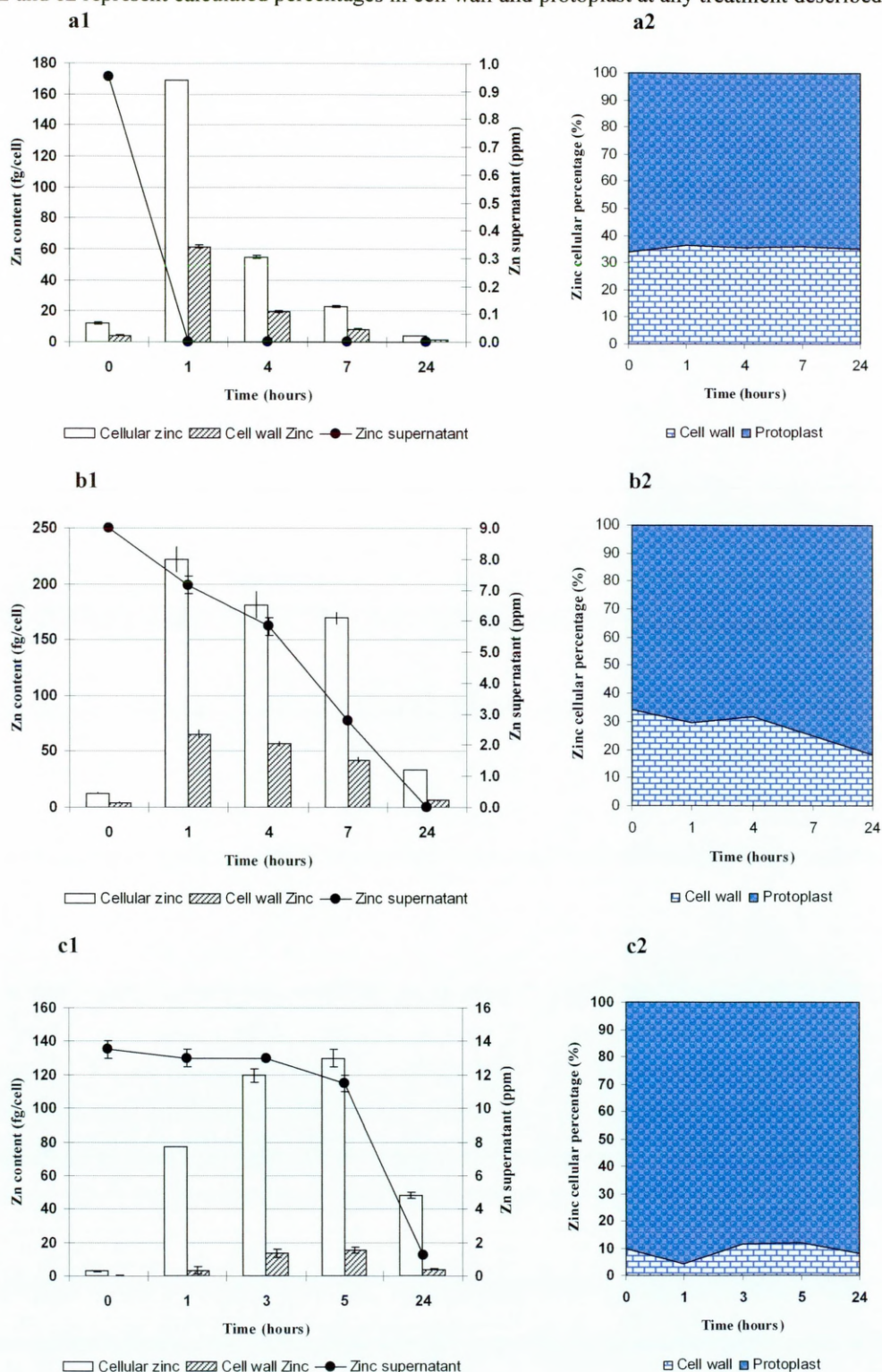
4.3 Results and discussion

Zinc in cell walls and protoplasts

Yeast cells growing in shake flasks, in malt wort are able to uptake all zinc in the first hour of growth at levels normally encountered in industrial media and at 25°C (see chapter 3). During growth, the localisation of tightly bound zinc to the cell wall was compared to the total zinc (free and bound). The mechanical breakage by acid-washed glass beads resulted in a suspension of cell walls (Fig. 2.6, Chapter 2) which were then washed several times with dH₂O or orthophenanthroline 0.1% to remove all loose zinc. The experiments with dH₂O showed that 30% of total cellular zinc was bound to the cell wall when cells were growing in malt wort at both normal and high levels of zinc (Fig. 4.1a; 4.1b). In the experiment with orthophenanthroline (Fig. 4.1c), this percentage was 5%. Orthophenanthroline is known to be a specific zinc chelator (Walker and Duffus, 1979) and this may have contributed to remove a higher percentage of zinc from the cell walls. In the latter experiment, only a high concentration (Zn at 13.5 ppm) of zinc was investigated since at normal concentrations, cell wall bound zinc was very low, below the AAS limit of detection (data not shown). Yeast cell walls did not appear to have a key role in zinc uptake, otherwise zinc percentage in the cell walls would have been expected higher than the range 5-30%, especially during the initial stages of fermentation. This result is in agreement with the work of White and Gadd (1987) who found most of the zinc (56%) in the vacuole, only 5% in the cytosol and the remaining part bound to other compartments, including cell walls. Free zinc was not visualised in the proximity of cell walls of yeast cells by using specific zinc probes (MacDiarmid, 2003).

In zinc uptake experiments by cells killed with heat treatment (see chapter 3), it was shown that zinc uptake was initially minimal and probably depended on the proportion of zinc bound to the cell wall. The bonds were loose and zinc was released back to the medium, probably because of damage provoked to the cell wall during the pre-heat treatment. It was concluded that biosorption by bond formation between zinc and the cell wall was not relevant during zinc uptake and an active metabolism was required. Moreover in the following stages of fermentation, the percentage of cell wall bound zinc remained constant indicating a secondary role of the cell wall. Although this experiment did not show precise cellular localisation of free zinc ions into the cell, it clearly indicated that zinc was rapidly accumulated inside the cell.

Figure 4.1 Zinc in yeast cell wall and protoplast during growth of lager strain in malt wort at various zinc concentrations Yeast cells of the lager strain B were collected, broken by glass beads and washed several times to remove loose zinc ions prior to nitric acid digestion. Pictures represent: cells grown in wort with Zn at 0.9 ppm after wash with dH₂O (a1), cells grown in wort with Zn at 9 ppm after wash with dH₂O (b1) and cells grown in wort with Zn at 13.5 ppm after wash with orthophenanthroline 0.1% (c1). Graphs a2, b2 and c2 represent calculated percentages in cell wall and protoplast at any treatment described above.



Intracellular zinc localisation by microscopic visualisation

A lager yeast strain (lager B) growing in complex media (malt extract broth) exhibited a high level of auto-fluorescence when exposed to wavelengths in the green range (530nm) (Fig. 4.2.a). This phenomenon probably depended on various cellular compounds fluorescing at the specific wavelength: NADH, riboflavin or the so called Schiff bases (Brunk and Terman, 2002). The level of auto-fluorescence was also very high in dead cells (Fig. 4.2.b) where these compounds are usually highly produced. Cells were grown up in EMM3 minimal medium (Fig. 4.2.c) in order to reduce the synthesis of these compounds and to diminish background autofluorescence, interfering with the zinc staining.

After one hour of growth, when intracellular zinc levels were very high, free zinc ions were clearly located into the yeast vacuole as shown in Fig. 4.3. MacDiarmid *et al.* (2003) observed similar zinc localisation by epifluorescent microscopy using two strains of *S.cerevisiae*: a wild type yeast strain and a *Zrc1* mutant lacking of the *Zrc1* gene. Both strains were previously starved of zinc, preloaded with a fluorescent dye (FuraZin-1 excited at 325 nm) and then exposed to high zinc levels (100 μ M). The mutant strain exhibited increases in the fluorophore signal but at a much reduced rate in comparison to the wild-type. The gene *Zrc1* was found to be required for sequestering zinc into the vacuole. Devirgiliis *et al.* (2004) also stained cellular zinc with Zinquin, a specific fluorescent dye which specifically binds zinc ions at nanomolar concentrations and produces fluorescence upon excitation with UV light. The concomitant staining of the vacuole and mitochondria, showed no correspondence between the areas of stained zinc and these cell compartments. Zinc was localised in small vesicles defined “zincosomes” (see Fig.1.3). The different results obtained by the two studies may have depended on the different specificity of the Zinquin probe compared to the FuraZin-1 (suitable to detect zinc ions in the range 0.1–100 μ M). The former may have been more specific to low levels of zinc and therefore more suitable to visualise only very low levels of zinc. Unfortunately, in the Zinquin work, no data are provided on the origin of the probe and related K_D , as indicator affinity of the dye to zinc.

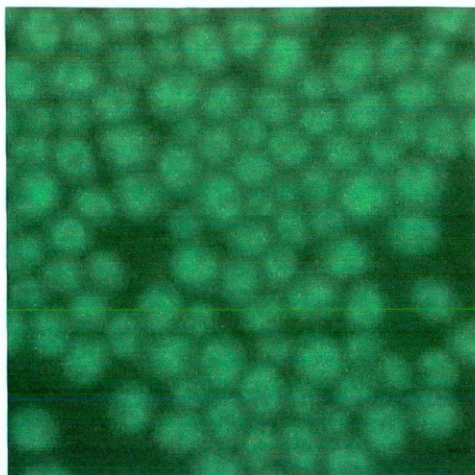
As proved in this study, zinc localisation in the vacuole extended the findings of the previous experiment, where the majority of zinc was found concentrated in the cells (70% to 95% depending on the cell washing solution). The yeast vacuole is a reservoir for several

nutrients (Lichko *et al.*, 1982) and vacuolar polyphosphate bodies have already been shown to be associated with zinc (Jones and Gadd, 1990). Zinc is probably stored in this organelle to become easily available when needed, more easily shared to the daughter during cell division or to maintain zinc homeostasis when zinc levels can be toxic to yeast cells (see Eide, 2003). This finding paves a new hypothesis on the mechanisms regulating the zinc distribution among population since zinc may be inherited during the budding process (see following paragraph on zinc distribution in yeast population), through cellular mechanisms of “proportional sharing”. The vacuole is actively divided between mother and daughter cells. This inheritance initiates early in the cell cycle and ends in G₂, just prior to nuclear migration (Weisman, 2003). The process begins with a portion of the vacuole extending into the emerging bud. This tubular-vesicular entity, the segregation structure, enables continued exchange of vacuole contents and therefore zinc between mother and daughter vacuoles.

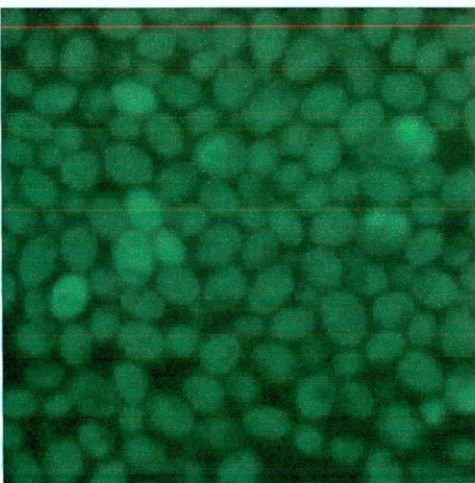
The use of zinc specific probes to stain yeast cells during any phase of the yeast growth may give a clearer picture of the zinc localisation during fermentation. Different dyes with diverse affinities to zinc (K_D) may be employed to visualise precise localisations of different levels of zinc. Attempts were also made to visualise zinc localisation during uptake at regular intervals, regulating the excitation dose in an economical way. Unfortunately, the zinc probes were destroyed very rapidly, by light-induced conversion of the probe material to a non-fluorescent compound (bleaching). This phenomenon does not allow the use of these dyes in traditional quantitative microscopy, where the intensity of the fluorescence is used to extract information about the local concentration of molecules that are labeled with fluorescent probes. The use of the flow cytometer overcame this problem since stained cells flowing through the laser were exposed to the specific wave-length for a very short time, avoiding any photo-bleaching effect (see following experiment). Unfortunately, dead cells would be unable to de-esterify the probe, a process required to activate the fraction of the dye able to form bonds with free zinc. Moreover, non-specific bonds are formed in dead cells, appearing highly fluorescing regardless their zinc content. As a result, the visualisation was not possible. Therefore, the use of this molecular probe is not indicated for studies on cells exposed to stress, where high percentages of dead cells may be expected.

Figure 4.2 Photo-bleaching of unstained lager yeast cells in various media Unstained cells of the strain lager B were visualised under fluorescent microscope using a green filter (530 nm) after growth in Malt Extract Broth (a) and in EMM3 minimal medium (b). Dead cells, previously grown in minimal medium and killed by heat treatment at 65°C for 1 hour were also visualised (c).

a



b



c

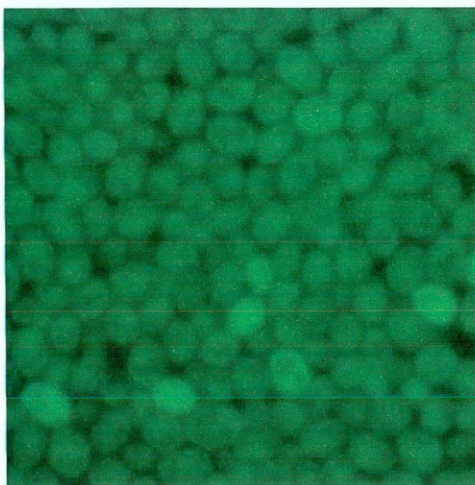
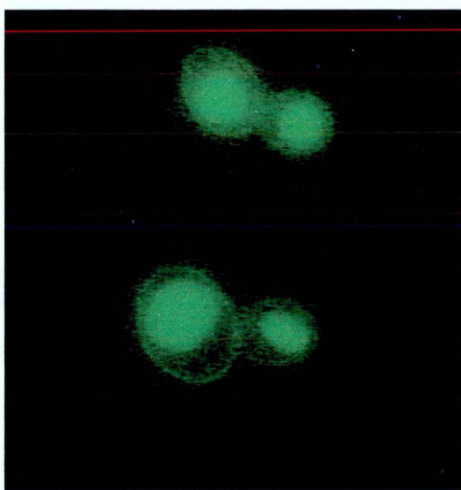


Figure 4.3 Free zinc ion and vacuole localisation in yeast cells. Yeast cells of the lager strain B were cultured in EMM3 medium (Zn at 10 ppm) and stained with both Fluo-Zn3 (for zinc visualisation) and Cell-Tracker B (for vacuole visualisation). Pictures were taken using a LEICA microscope under bright field (a), using a green filter (b) and a blue filter (c).

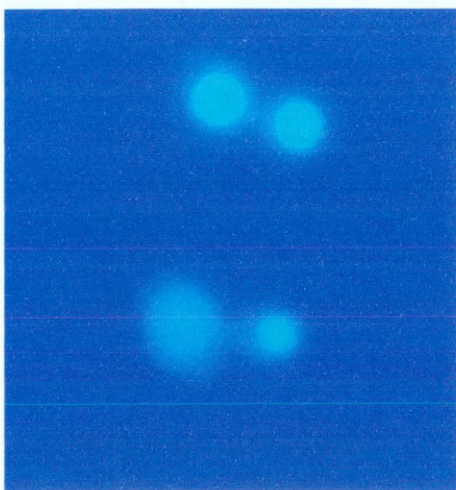
a Phase contrast (control, no stain)



b Fluo-Zn3 stained cells (zinc visualisation)



c Cell-Tracker B stained cells (vacuole visualization)



Zinc distribution in yeast cell populations

Studies on zinc distribution among yeast cell populations were carried out by sampling cells of different size. Cells were harvested after 14-16 hours of growth, in late exponential phase when cell populations were not homogeneous with regard to cell size. Cells of different size were collected after centrifugal separation in lactose gradient solutions (Figs. 4.4; 4.7), following the principle that big size cells (mother cells) are in equilibrium with a more concentrated solution while small cells (daughter cells) are in equilibrium with a more diluted solution. *S. cerevisiae* is not able to utilise the sugar lactose and despite its concentration being high (from 15 to 30%), cells were not exposed to osmotic stress since the sampling was very quick, avoiding any alteration of cellular status and intracellular zinc content.

Zinc cell content was analysed, following growth, in two different conditions: after growth in normal (0.25 ppm) and high (24 ppm) zinc concentrations. The former condition is normally encountered in brewing wort, the latter is unusual and can be encountered only after addition of zinc salts. Such a high concentration was not toxic to the brewing yeast strain employed in this study (see also chapter 5).

Zinc distribution in yeast cell populations in condition of excess zinc (24 ppm)

The experiment with zinc in excess was useful to determine an ideal situation of zinc repletion. When cells were grown in high zinc medium (24 ppm), for 16 hours, intracellular zinc concentrations remained constant in daughter (78 and 81 fL) and mother (112 and 117 fL) cells (Fig. 4.5; 4.6). They took the bioavailable zinc, not only prior to cell division but also after separation. The uptake was uninterrupted until cells reached the “zinc saturation” point which was obviously related to the size of the cells. When saturation was gained, zinc cellular concentration was the same and zinc cell content was correlated to the size of the cells (Fig. 4.6).

Zinc distribution in yeast cell populations in conditions of normal zinc levels (0.25 ppm)

Studies with cells growing in normal zinc medium (0.25 ppm), were necessary to determine the exact proportion of zinc shared from mothers to daughters during cell division. Since the level of intracellular zinc was very low, AAS could not be employed for zinc cellular

content analyses. The use of specific zinc fluorescent dyes and the determination by flow cytometry of the fluorescence emitted by cells of different sizes, gave an estimate of the intracellular free zinc. Fig. 4.8 shows that the intensity emitted by large cells (132 fL) was higher than that emitted by smaller daughter cells (57 fL). The fluorescent cells, counted during the same period of time (60 sec), of the same cell number and with the same flow rate were 2604 for mothers compared to 1886 for daughters. These data did not show differences in the emitted fluorescence but more mother cells were fluorescing than daughters.

The different results between the two experiments may have depended on the initial levels of zinc in the medium. At 0.25 ppm of zinc, zinc ions were shown to be completely uptaken from the medium after the first hour (see chapter 3). During cell division, daughter cells received zinc from their mothers. The same cells were not able to uptake further zinc from the medium, because it was not available. With growth, cells became bigger and the zinc concentration became progressively less. This may explain the reason why after some cell cycles, the intensity of the emitted fluorescence was equal for all cells. A part of daughter cells were not fluorescing, the reason being that during budding process some cells may have received very low zinc levels from their mothers, not detectable by the flow cytometer. It is conceivable also that part of zinc was stored intracellularly in cells, not as free ions but after having formed bonds with cellular compounds. Of course bound zinc ions were not detectable by the zinc fluorescent probe which was specific only for free zinc ions. It would have been interesting to have available an instrument to detect total zinc with a lower limit of detection than AAS (e.g. Inductively Coupled Plasma Source Mass Spectrometry or Stripping Voltametry).

The findings of this experiment may have practical applications in industry. In lager brewing, in cylindro-conical fermenters, at the end of fermentation, cells flocculate at the bottom of the fermenter forming the yeast cone. Such a high density of cells is kept until the beer level of diacetyl (a beer off-flavour) has been reduced to commercially acceptable levels. Since heterogeneity in yeast age, viability and fermentation performance after analyses of specific environmental parameters and physiological factors were found at various fractions of the yeast cone (Powell *et al.*, 2004), different levels of zinc may also be expected. In a brewery, at end of fermentation and prior to yeast repitching or storage, the

practice to remove part of the yeast cone may deplete yeast biomass of an important reservoir of zinc. In this regard, it would be interesting to determine cell size and zinc levels of various fractions of the yeast cone, in industrial scale vessels, after fermentation.

Zinc distribution in yeast cell populations in conditions of excess zinc (24 ppm)

Figure 4.4 Cell size of the lager strain B grown in malt wort (Zn at 24 ppm) for 16 hours after sampling from a lactose gradient solution. Cells of the strain lager B were grown up in malt wort (Zn at 24 ppm), in shake flasks for 16 hours, harvested and separated using a lactose gradient solution. Two samples were taken from the top of the gradient solution: 78 fL and 81 fL and from the bottom: 116 fL and 112 fL. The mean volume population was 155 fL (seed). Cells were analysed by Coulter Multisizer

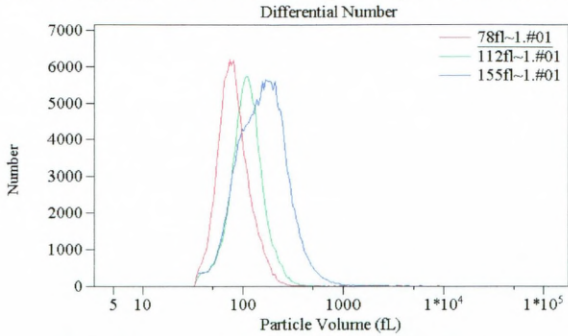


Figure 4.5 Zinc cell content and concentration in brewing strain cells of various size. Yeast cells of various sizes were found to have same Zn concentration. These finding were different from earlier work (see Zinc uptake by various yeasts in Malt Extract Broth medium, paragraph 3.5). Cells of various yeast strains and of different sizes were found to have also different Zn concentration (e.g. ale strain big cells with lower Zn than *K. marxianus* small cells).

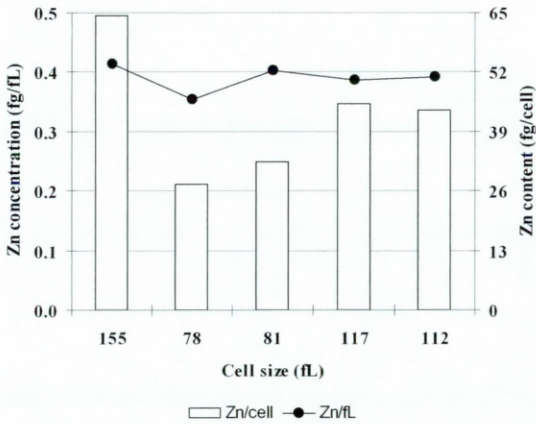
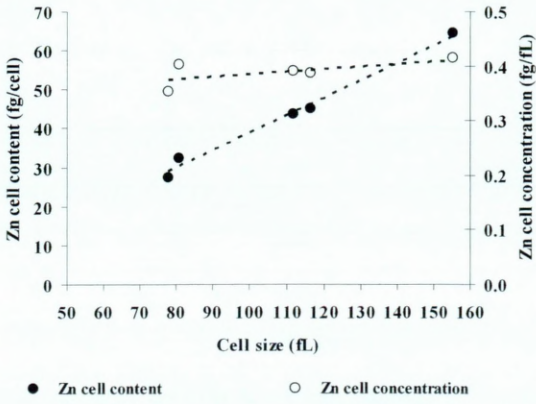


Figure 4.6 Relation between zinc cell content and concentration in brewing strain cells of various size. Zn cell content was proportional to the size of the yeast cells. Zn cell concentration was constant indicating and equal distribution of zinc between mother and daughter cells in condition of excessive zinc.



Zinc distribution in yeast cell populations in conditions of normal zinc levels (0.25 ppm)

Figure 4.7 Cell size of the lager strain B grown in MEB (Zn at 0.25 ppm) for 14 hours after sampling from a lactose gradient solution. Cells of the lager strain B were grown up in malt extract broth, in shake flasks for 14 hours, harvested and separated using a lactose gradient solution. Samples were taken from the top of the gradient solution: 57 fL and from the bottom: 132 fL. The mean volume population was 166 fL.

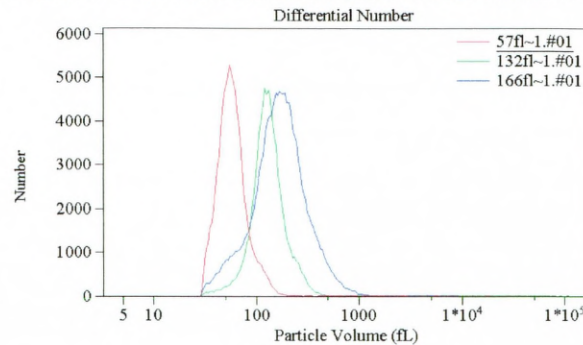
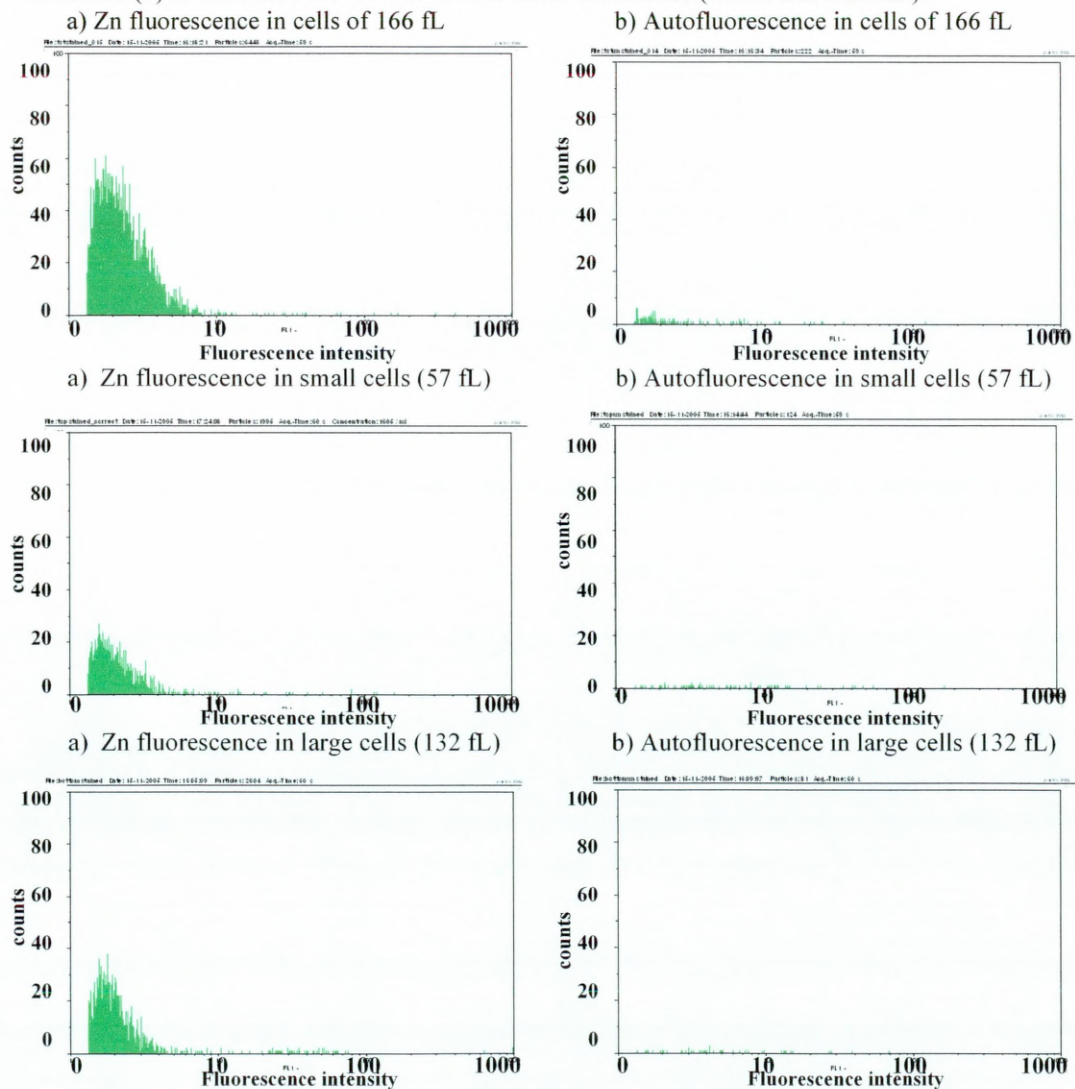


Figure 4.8 Fluorescence in brewing yeast cells of different sizes analysed by flow cytometry Cells were stained with Rhod-Zn 1 and run by a Partec Cyflow flowcytometer. Stained cells (a) were compared to unstained (b) to determine the level of cellular autofluorescence (which was minimal).



4.4 Summary

The results of the research presented in this chapter showed that:

- The yeast cell wall does not play a key role in zinc uptake during fermentation.
- Zinc is rapidly stored into the vacuole and shared from mother to daughter cells during cell division.
- In brewing and bioethanol industries, where yeast biomass is recycled for several fermentations, particular attention must be paid to the practice of removing part of the yeast crop. This may lead to the depletion of an important reservoir of zinc from the yeast biomass.

Chapter 5

Influence of zinc on yeast fermentation performance

5.1 Introduction

In alcohol production processes by fermentation, a major effort is made to keep yeast cells under appropriate physical, chemical and nutrient conditions in order to minimise cell growth and stimulate metabolic pathways leading to optimal alcohol production. However, in laboratory conditions, growing cells produce alcohol 33 times faster than non-growing cells (Ingledew, 1999). Compromise efforts are made to keep yeasts under conditions that do not lead to low growth rates or to cell death. Control and management of micronutrient levels, and particularly of zinc, are vital for the correct progress of fermentation and to encourage a predominantly fermentative, rather than respiratory, mode of metabolism in the yeast strains employed in alcohol production.

In the brewing industry, initial zinc concentrations may be determined in malt wort, throughout the fermentation process and in the final beer. Zinc supplements are commonly implemented when zinc levels are very low to avoid sluggish and incomplete fermentations. Lack of zinc in malt wort has been fully documented (Daveloose, 1987; Jacobsen *et al.*, 1982; Kreder, 1999; Jacobsen *et al.*, 1982; Jacobsen and Lie, 1977a, 1979) and found dependent on the technological process employed to prepare the malt wort. Moreover, although present, some of the zinc may be not bioavailable to the yeast cells. In the literature, various levels of zinc have been proposed for optimal fermentation performance in brewing: between 0.1 and 0.15 ppm (Bromberg *et al.*, 1997), 0.18 ppm (Helin and Slaughter, 1977b) and between 0.05 and 0.30 ppm (Jacobsen *et al.*, 1981). Zinc cellular requirements are yeast strain dependent (Rees and Stewart, 1998) but also the malt wort quality and the fermentation conditions are most probably determining differences in zinc demand by yeast cells. Zinc affects the flocculation of only some ale strains and at the relatively high concentrations of 2.6 ppm (Raspor *et al.*, 1990). During fermentation, zinc may interact with other cations such as calcium, involved in the flocculation process, thus promoting this phenomenon. The hypothesis that zinc is involved in flocculation is also

supported by zinc limitation studies in aerobic continuous culture (De Nicola and Hazelwood, unpublished). In these studies, the gene *MUC1*, was found 50 and 30 times more up-regulated in zinc-limitation conditions compared to, respectively, carbon and nitrogen limitation albeit in a non-flocculent strain. This gene is involved in yeast flocculation (Guo *et al.*, 2000) through the synthesis of the protein Flo11p, member of the second group of the Flo family proteins, uniformly localised around the cell surface of the haploid cells but only in few surface areas of diploid yeast cells. When overexpressed, Flo11p can function in mating, invasion, filamentation and flocculation. Genes encoding for these proteins have a similar overall domain structure, have considerable diversity in amino acid sequence and can compensate for other *FLO* genes in diverse morphogenic events: flocculation, mating, haploid invasion, and filamentation. Zinc depleted cells were found to form clusters (Obata *et al.*, 1996). Positive influences of zinc on the synthesis of esters and higher alcohols as well as the decrease of acetaldehyde levels have been widely documented (Hodgson and Moir, 1990; Seaton *et al.*, 1990; Skanks *et al.*, 1997; Quilter *et al.*, 2003)

In the wine-making industry, it is unusual to carry out analyses of zinc levels since the metal concentration in grape juice ranges from 0.04 to 7.8 ppm with an average of 0.90 ppm (Cabanis and Flanzy, 1999) and this is usually deemed satisfactory for the progress of the fermentation. In wine-making, research on zinc interaction with yeasts is mostly performed by companies involved in the commercialisation of yeast supplements. Commercial preparations (e.g. “*Goferm*”, from Lallemand Ltd.), based on nutrients such as organic and inorganic nitrogen, fatty acids, sterols, vitamins and mineral salts, including zinc, are usually added during yeast rehydration and propagation to ensure that yeast cells are supplemented with satisfactory levels of nutrients prior to cell inoculation. These actions aim to guarantee that yeast cells are healthy and active from the early stages of the fermentation. Unfortunately, there are no previous studies available on the influence of zinc with wine yeast strains and most of the research was focused on agronomic studies of the vineyard soils or grape fertilizers based on zinc (Christensen, 1980; Christensen and Jensen, 1978).

In the bioethanol (fuel alcohol) industry, as in wine-making, zinc levels in beet molasses (40 ppm) and in sugar cane molasses (13 ppm) (Curtin, 1973) are usually abundantly available to the yeast cells. However, studies on zinc supplements in molasses using a selected distilling yeast strain (Dr. K. Leiper, personal communication) proved that the addition of 0.5 ppm of zinc was beneficial and increased the amount of ethanol production by about 1% at specific gravities higher than 1096. However, these fermentations did not attenuate fully and increasing amounts of residual sugar, mostly fructose, remained. At lower specific gravities (e.g. 1088), there was no improvement in ethanol production, mainly as the unsupplemented fermentations attenuated fully anyway. Generally speaking, in the bioethanol industry, the addition of fine chemicals to fermentations would not be financially attractive and little research is carried out on zinc supplements.

5.2 Experimental

Experimental aims

The aim of the research presented in this chapter was to study the influence of zinc on fermentation performance of brewing and wine-making strains of *S. cerevisiae* in order to determine the concentration of zinc giving fastest fermentation rates in malt wort and grape must respectively. For brewing yeast, the influence of zinc on beer flavour profile was also investigated to establish the concentration of zinc providing appreciable changes in volatiles and flavour profiles.

Experimental approach

The influence of various zinc levels on yeast fermentation performance was investigated on a lager yeast strain (lager B) and two wine yeast strains: A and B, respectively L-2056 and L-2226. Experiments were performed in small-scale Imhoff conical vessels to simulate wine making and brewing processes. Studies on the lager brewing strain were extended to industrial scale, in the pilot plant of a brewery with the intention of completing the fermentation performance data with the analyses of beer flavour profiles.

Experimental procedure

Studies on zinc influence on brewing fermentations (small scale conical vessels)

Seed cultures of the lager strain B were prepared in malt wort pre-treated with BioRad Chelex (see paragraph 2.2.2). Divalent cations and trace elements were re-established using the same concentrations as in EMM3 minimal medium with zinc concentration at 0.10 ppm. Cells were grown in shake flasks at 25°C, 180 rpm, for 48 hours and transferred to 14°C for 8 hours prior to the beginning of the experiment.



Preparation of the malt wort according to the method described in paragraph 2.2.3.
Pre-aeration of the malt wort for 2 hours at 14°C



Inoculation of 5×10^6 cells/ml in Imhoff conical vessels (1L) at 14°C with quick mix of the cell population. Zinc levels were adjusted with zinc acetate to have respectively 0, 0.05, 0.12, 0.48, 0.82, 1.07, 4.85, 10.8, 23 ppm of Zn



Sampling at 0, 1, 6, 24, 48, 96, 144, 192, 264 hours
for: number of cells in suspension, size of the yeast cone, cell size, viability, metal ion concentration in medium and metal ion cell content, free amino nitrogen (FAN), specific gravity, ethanol concentration and membrane fluidity (Generalized Polarization)

Studies on zinc influence on brewing fermentations (pilot plant)

Seed culture of the lager strain B collected from the storage tank of the brewery, after one fermentation



Inoculation of $\sim 10^7$ cells/ml in pilot plant fermenters (200 L) at 11°C
Cells were added to the fermenter during the process of filling with malt wort.
Zinc levels were adjusted with zinc sulphate to have respectively 0, 0.5, 1, 5 and 10 ppm of Zn



Sampling at 0, 18, 41, 65, 87, 138, 161 and 186 hours
For: cell counts, dry weight, Zn cell content, ethanol and specific gravity, turbidity and pH.
Volatile analyses were kindly provided by the Quality Control Department of Heineken Supply Chain (Zoeterwoude, the Netherlands).

Studies on zinc influence on wine fermentations (small scale conical vessels)

Seed culture of the wine strains A and B were prepared in grape juice
(zinc concentration at 0.10 ppm).

Cells were grown in shake flask at 25°C, 180 rpm, for 24 hours

↓

Inoculation of 5×10^6 cells/ml in Imhoff conical vessels (1Lt) at 25°C
with quick mix of the cell population. Zinc levels were adjusted with zinc acetate to
have respectively 0.9, 1.5, 2.5, 14 and 26 ppm of Zn

↓

Sampling at 0, 5, 24, 48, 72, 96, 120 hours
for: number of cells in suspension, size of the yeast cone, cell size, viability, metal ion
concentration in medium and ethanol concentration

5.3 Results and discussion

Studies on zinc influence on brewing fermentations (small scale conical vessels)

In brewing simulated small scale fermenters, yeast cells of the lager strain B were able to uptake the zinc from the media, with similar patterns described for the shake flask experiments (see chapter 3). The time required for complete removal of zinc from the medium depended on the initial zinc concentration. For example, at 0.48 ppm of extra-cellular zinc, all zinc was taken up in the first 6 hours (Fig. 5.1a). At higher concentrations, the removal was gradually delayed: at 10 ppm only after 4 days and at 23 ppm residual zinc was still present in the medium after 11 days (Fig. 5.1b). As a result, there was a big difference in terms of mean zinc content in suspended cells (Figs. 5.2a; 5.2b) with values above 1300 fg/cell in the medium with 23 ppm of zinc, compared with the initial 1.22 fg/cell of the seed culture. At higher concentrations, during cell division, zinc was diluted between mothers and daughters (see chapter 4) and the latter continued to uptake further zinc from the medium of growth. This situation can be compared with the flow cytometer analyses described in chapter 4 “Zinc distribution in yeast cell populations in conditions of excess zinc”. At low zinc levels (from 0 to 0.82 ppm of zinc), intracellular zinc was higher in the first hour compared to the end of fermentation. After the cell divisions occurring in the first 6 hours, cells were not able to uptake further zinc (see also chapter 4 “Zinc distribution in yeast population in condition of normal zinc levels”). The longer time required for complete zinc uptake in brewing small scale experiment may have depended on the low temperature of fermentation (14°C) and the cell heterogeneity in the medium due to the absence of agitation of the conical vessels. Oxygen levels in the medium at the initial stages were high, due to the pre-oxygenation of the malt wort prior to inoculation. Only when cells actively fermented, the generated carbon dioxide contributed to mix up yeast cell population in the growth medium. Most likely oxygen did not play a role on zinc uptake.

In similar experiments, in 2 L static fermenters, Mochaba *et al.* (1996) described different patterns occurring during zinc uptake in malt wort. In the first 4 hours of fermentation, at 0.75 ppm of zinc, all zinc was uptaken from the medium and levels fluctuated during fermentation to reach maximum intracellular zinc levels at the end of fermentation. After repitching in fresh malt wort, yeast cells released zinc ions back to the medium to uptake them again subsequently. Overall, yeast cells performed poorly and high was the sensibility

to relatively low levels of extra-cellular zinc (0.75 ppm). The different zinc uptake kinetics and the high sensitivity to zinc ions were most probably dependent on the yeast strain employed in these experiments. In this Thesis, high zinc content was not accompanied with loss of viability which remained above 95 % in all the conical vessels (data not shown). Initial manganese levels in malt wort were 0.20 ppm. This concentration was half of the 0.40 ppm reported by Jones and Greenfield (1984) to be necessary to support levels of zinc above 2 ppm. This value may be again strain dependent and the lager strain used in this experiment may have high zinc tolerance. Since malt wort medium is rich in nutrients, other metals may have influenced yeast cell tolerance to zinc. For example, Bromberg *et al.* (1997) found that zinc requirements were higher in poor quality malt wort indicating evidence of interaction between trace metals and the zinc capacity to replace other trace metals.

Cell growth was not affected by various zinc levels. This is in agreement with the work of Jones and Greenfield (1984) and Bromberg, (1997). At the end of fermentation, cells flocculated at the bottom of all the conical vessels forming the yeast cone, with the exception of the zinc free medium where cell numbers in suspension remained very high (Figs. 5.3; 5.4). This was probably the consequence of the turbulence produced by the carbon dioxide, still present after 11 days at very low zinc concentrations when sugars were not completely utilised (see also Fig. 5.6a). Overall, various zinc levels did not influence yeast flocculation and the uptake of other divalent cations which may play a role in the phenomenon of flocculation (see Figs. 5.3, 5.5). Moreover, throughout the fermentation, calcium, magnesium or manganese residual concentrations were not affected by various zinc concentrations (Fig. 5.7a; 5.7b; 5.7c), thus demonstrating the absence of interaction among these cations to occupy the cell surface sites involved in flocculation. The rapid ion uptake appeared specific only to zinc. This was previously shown in shake flask uptake studies of four different industrial strains in malt wort (Fig. 3.3) and on the influence of calcium on zinc uptake by a lager strain (Fig. 3.10). Taylor and Orton (1973) demonstrated that flocculation occurred only at concentrations above 6500 ppm and pH 7.5 and Raspor *et al.* (1990) showed that only some ale strains may flocculate *in vitro* at 2.6 ppm of zinc. Lager strains were shown not to be affected by the presence of zinc in terms of flocculation and

this phenomenon was explained by possible differences in cell surface structure of ale brewing strains.

At the end of fermentation, there was no difference in mean cell volume between cells in suspension and in the yeast cone at various zinc concentrations (Fig. 5.5). In preliminary experiments, yeast cell size was investigated at various fractions of the yeast crop formed at the bottom of the fermenter and homogeneity was observed with regards to cell volume. This observation was in contrast with previous studies of Powell *et al.* (2004) who found yeast cell heterogeneity at various fractions of the cone in 2000 hL brewing fermenters. Since the volume of the fermenters used in this Thesis was only 1 L, the carbon dioxide generated during fermentation may have kept the cell population continuously mixed, altering the distribution of the cells in the yeast cone.

Zinc concentrations in the range 0.48 to 1.07 ppm appeared to be the optimal in terms of yeast fermentation performance and this was supported by the data of both sugar utilised (Fig. 5.6a) and ethanol produced (Fig. 5.6b). Higher metabolic activity of the yeast cells in this range of zinc was also confirmed by the free amino nitrogen data (FAN) (Fig. 5.6c). FAN is a measurement of the low molecular weight nitrogenous substances, mainly amino acids, which are needed to support yeast growth and metabolism (Briggs, 2004). Above 0.05 ppm zinc, the rapid uptake of free amino nitrogen was certainly a result of a more active cellular metabolism. At lower initial zinc concentrations and particularly at 0 ppm of zinc, residual sugars were still high and the fermentation rate was strongly delayed. FAN levels were also higher indicating an inefficient metabolism of the cells (Fig. 5.6c). A milder delay in fermentation rate was also shown at higher concentrations (5, 10 and 23 ppm) but after 264 hours, the final ethanol yield was the same as in the range 0.48-1.07 ppm. Cells used as seed culture in this experiment were grown up in low zinc medium (0.10 ppm) to lower the initial zinc cell content and to better appreciate the effect of various zinc malt wort concentrations on fermentation. Cells preconditioned in a medium with more zinc and thus with higher initial zinc cell content, may have performed very well also in zinc free medium as in the pilot plant experiment (see following paragraph). Results indicated that the range 0.48 to 1.07 ppm of zinc was optimal for fermentation performance of the lager yeast strain B. Due to the small volume of the fermenters employed in this study, additional information on the influence of zinc on volatile compounds was not possible. The small scale brewing-simulated fermentations offered the great advantage to

better control parameters such as zinc levels in each single vessel (due to the acid-washing procedure) and to avoid possible zinc trace contamination from the walls of the vessels. The transparency of the Imhoff fermenters allowed a better estimation of the yeast cone formation, visible from outside. Unfortunately, due to the small volume of the vessels (1L) many analyses were not possible (dry weight, haze, volatiles, etc) and the CO₂ formed during fermentation mixed the cells in the small yeast cone. As a result, accurate analyses of size and zinc cell content of the yeast cone at various fractions were not possible.

Membrane fluidity of the *S. cerevisiae* lager strain B was analysed by measuring Generalized Polarization (GP) of the membrane-localising fluorescent probe Laurdan. Zinc levels impacted upon membrane fluidity (Fig. 5.8) which varied with culture age and ethanol accumulation, as well as in relation to cellular zinc levels. When different levels of zinc addition were compared on each day (Fig. 5.8), some variation was seen indicating a complex relationship between membrane fluidity and initially added zinc levels. However, for each curve most points fell within 10% of the zero zinc GP value. The GP of the cell membranes slightly increased (indicating a fluidity decrease) until the 4th day of fermentation and then gradually decreased throughout the remaining period. When GP values and ethanol levels were averaged for each day over all fermenters (containing the different initial zinc concentrations), compared to an average GP of 0.4176 (when ethanol was less than 1%) after 1 day, there was a 9 % GP increase (when ethanol was around 2.5%) on day 4, and thereafter decreased GP of 10% on day 6 and 24% on day 8 (when ethanol levels averaged around 4 and 5%, respectively). By the end of fermentation at day 11, around 6% ethanol had accumulated, with a 31% decrease in GP in suspended cells and 45% decrease in sedimented cells. This indicated that membrane fluidity changed with cell aging and with nutrient availability. The lower GP values indicated higher membrane fluidity, which likely reflected the higher ethanol levels as fermentation progressed, placing the cells under ethanol stress. At the end of fermentation, cells remaining in suspension had higher GP values than cells in the yeast crop, which may relate to higher stress status of cells in the crop and the locally increased ethanol concentrations. In pilot plant experiments, cells in the crop were also found to have lower content of zinc and this may be related to the higher sensitivity to the stress conditions encountered in the yeast crop (see also comments on “Studies on influence of zinc on brewing fermentations, pilot plant”).

Figure 5.1 Zinc supernatant levels during fermentation of a brewing yeast strain in 1 L conical vessels with variable zinc concentrations. Yeast cells of the strain lager B were inoculated in Imhoff conical vessels, in malt wort with altered zinc levels. Fermentation was carried out for 264 hours (11 days). Zn residual levels in supernatant were analysed during fermentation in the range from 0 to 1.07 ppm of Zn (a) and from 4.85 and 23 ppm of Zn (b). For clarity, standard deviations were not included in the graphs of this chapter.

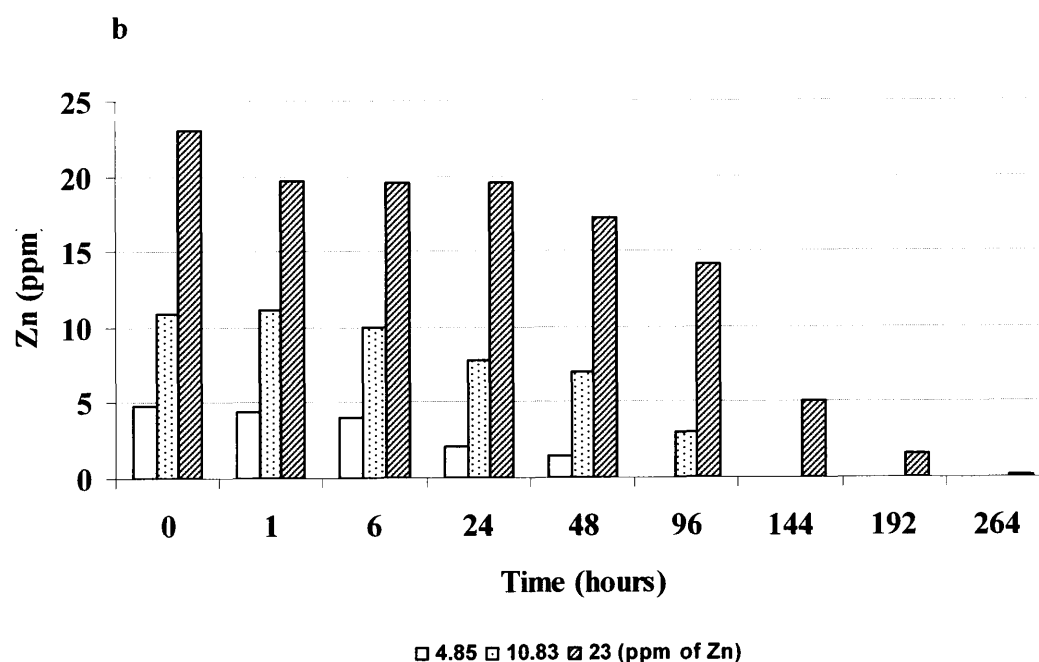
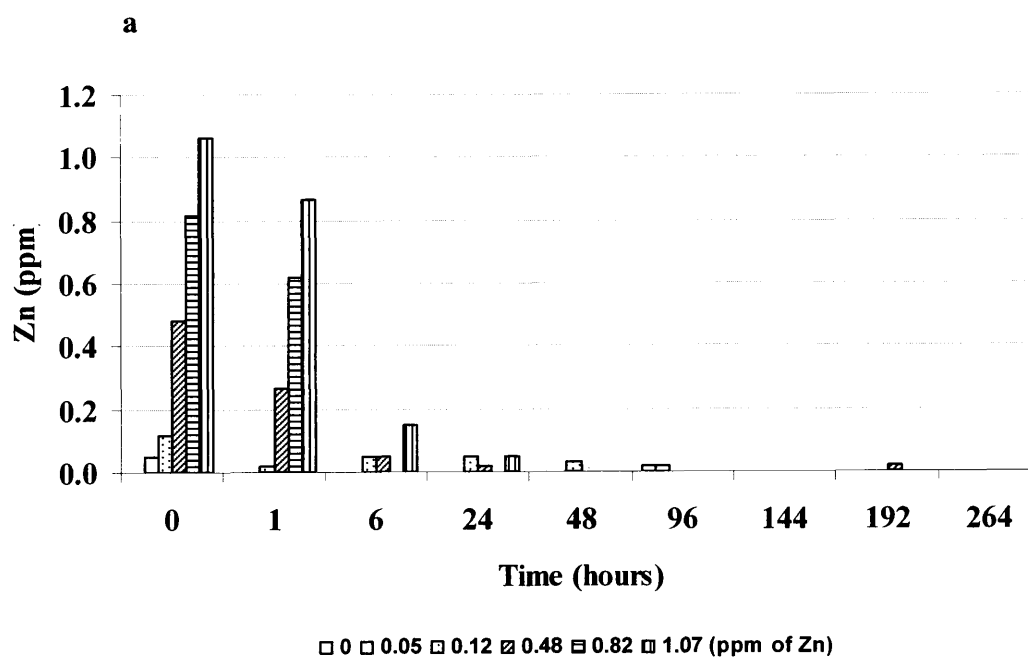


Figure 5.2 Zinc cell content of a brewing yeast strain during fermentation in 1 L conical vessels with variable zinc concentrations. Yeast cells of the strain lager B were collected during fermentation from the middle of the conical vessels, at 600 ml level indicated on the fermenter (at time 1h) and at the bottom of the fermenter (at time 264 h), in the yeast cone. Graphs represent the analyses performed, respectively, in the conical vessels with Zn in the range from 0 to 1.07 ppm (a) and in the range from 4.85 to 23 ppm (b). While the time 1 hour indicates Zn content of cells in suspension, the time 264 hours indicates Zn content of cells in the yeast cone. At the end of fermentation, few cells were left in suspension. Therefore, cells in the yeast cone were considered more representative of the whole cell population.

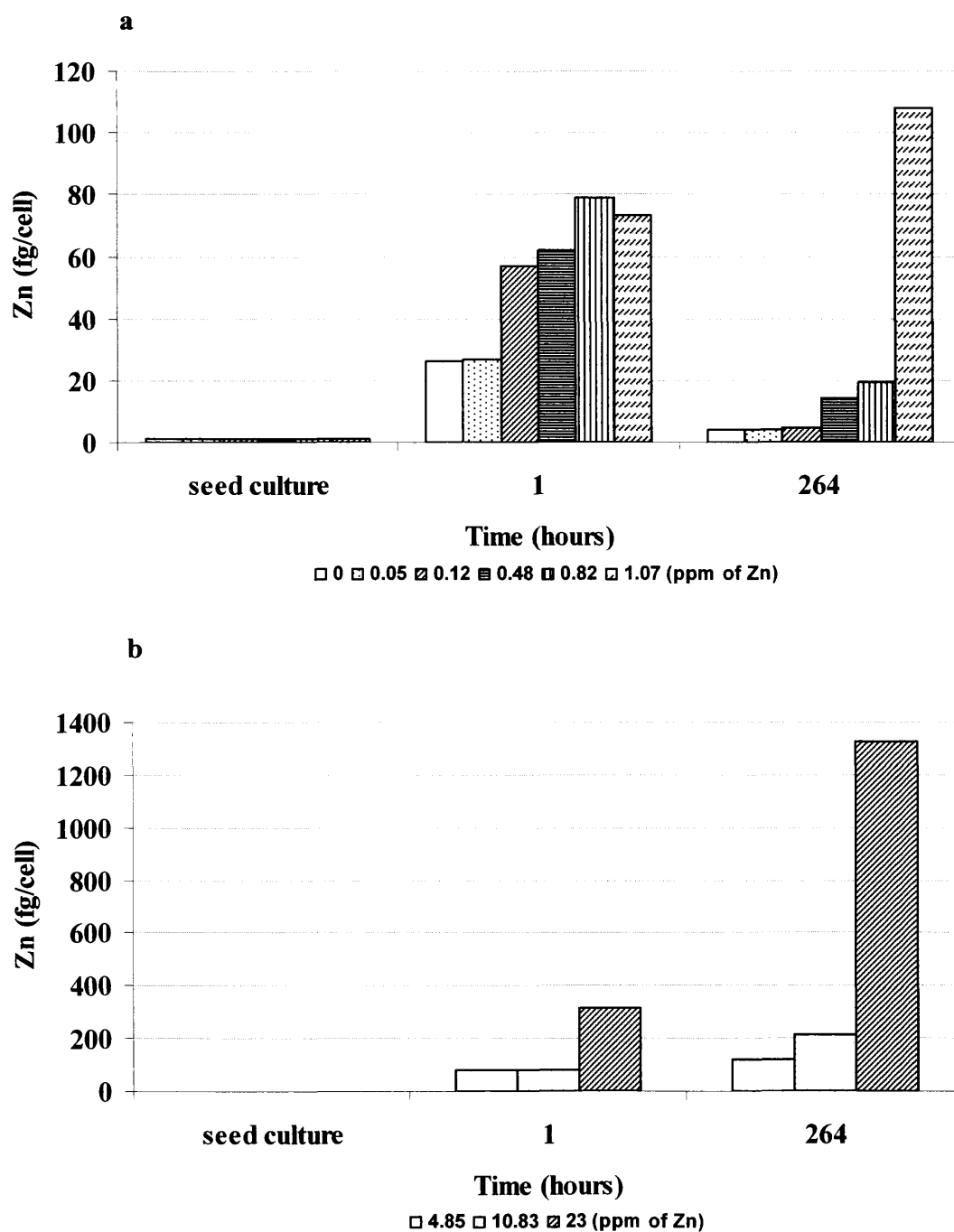


Figure 5.3 Yeast cone size of a brewing yeast strain during brewing fermentation in 1 L conical vessels
The size of the yeast cone formed by sedimented (floculated) cells (of the lager strain B) was regularly checked during fermentation as indication of cell growth and flocculation. In zinc free malt wort, yeast cells did not flocculate. This may have depended on the high levels of carbon dioxide still present after 11 days, due to the slow fermentation of sugars. For clarity, the figure reports only six of the nine concentrations of zinc studied, without standard deviations.

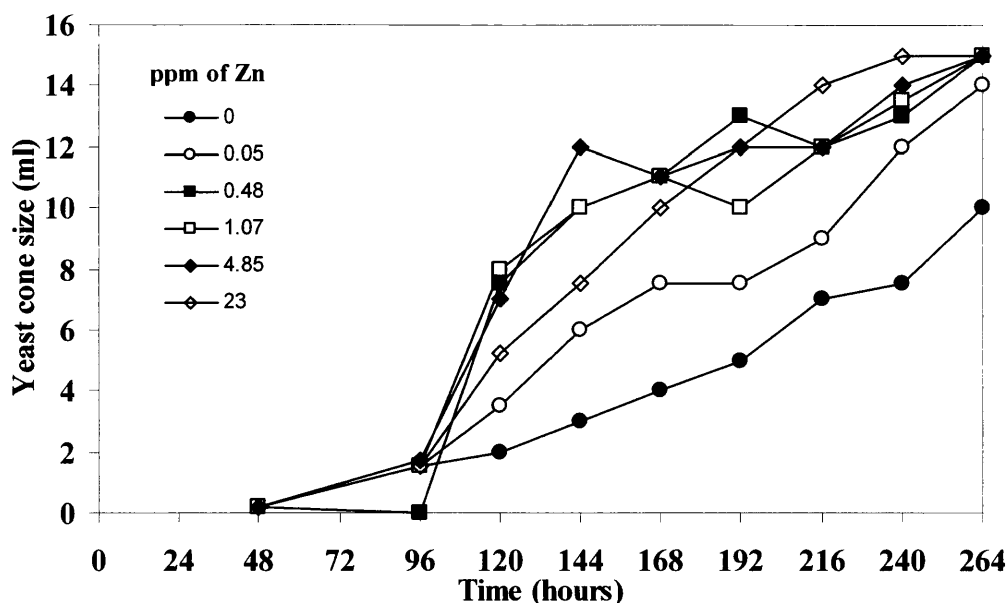


Figure 5.4 Growth of a brewing yeast strain during fermentation in 1 L conical vessels
Yeast cells of the lager strain B were sampled from the middle of the fermenter and counted by haemocytometer to determine, together with the yeast cone size analysis, the level of cell growth and flocculation. Suspended cell number at 0 ppm was consistently higher compared with other concentrations. This was probably due to the slow fermentation occurring in zinc depleted medium.

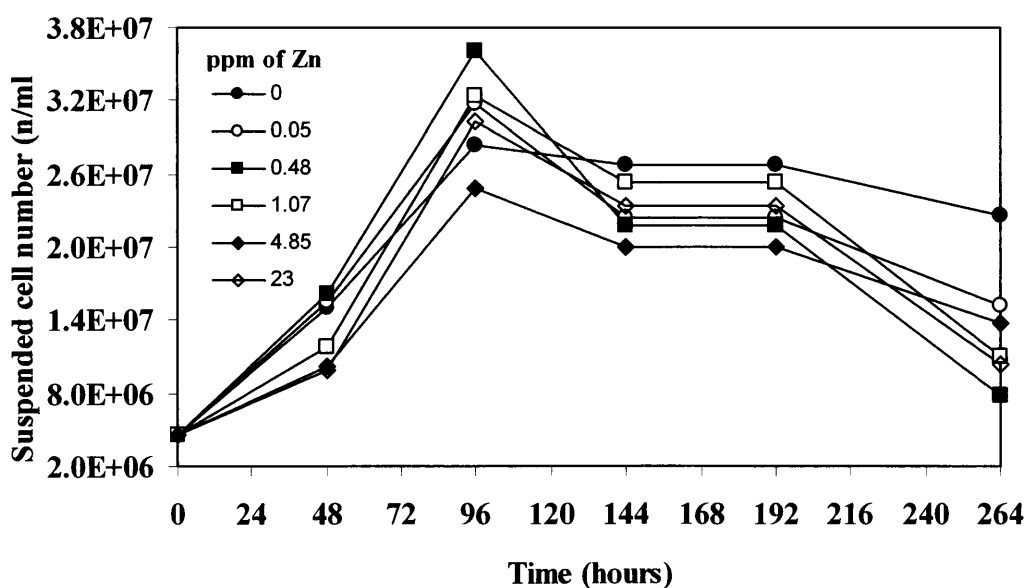


Figure 5.5 Size of suspended and precipitated cells of a brewing yeast strain at the end of fermentation in 1 L conical vessels The mean volume of cells in the yeast cone and in suspension was analysed to determine the influence of zinc on the mean cell size. In preliminary experiments, no major differences were observed between cells collected at various fractions within the yeast cone.

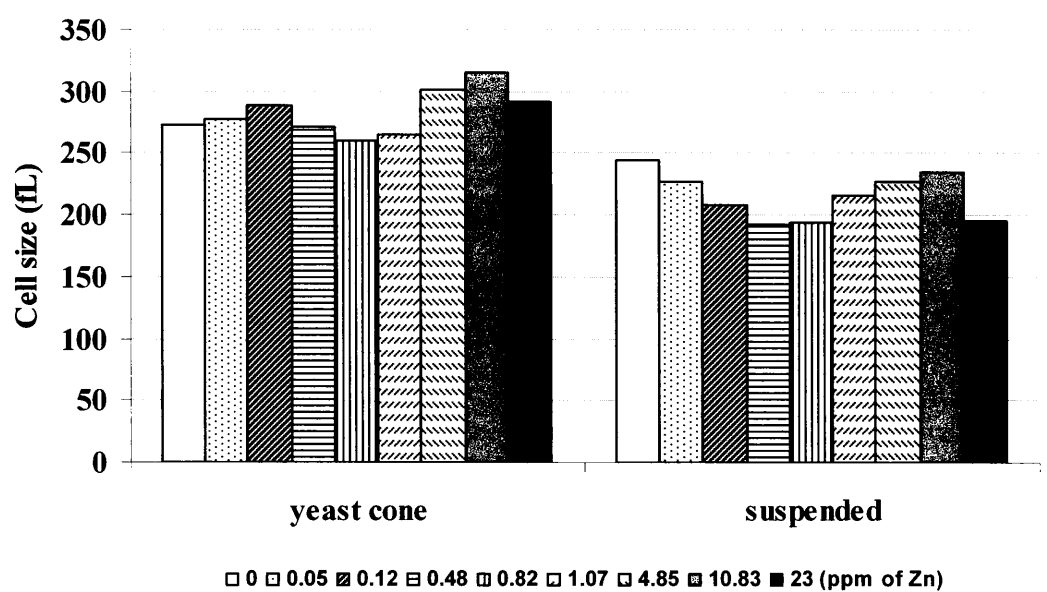


Figure 5.6 Specific gravity, ethanol and free amino nitrogen levels during fermentation of brewing strain in 1 L conical vessels. Fermentation performance was evaluated by analysing specific gravity (a), ethanol (b) and free amino nitrogen levels (c) at regular intervals. The symbols indicate the following Zn concentrations (ppm): 0 (□), 0.05 (▤), 0.12 (▥), 0.48 (▧), 0.82 (▨), 1.07 (▩), 4.85 (▪), 10.83 (▫), 23 (■).

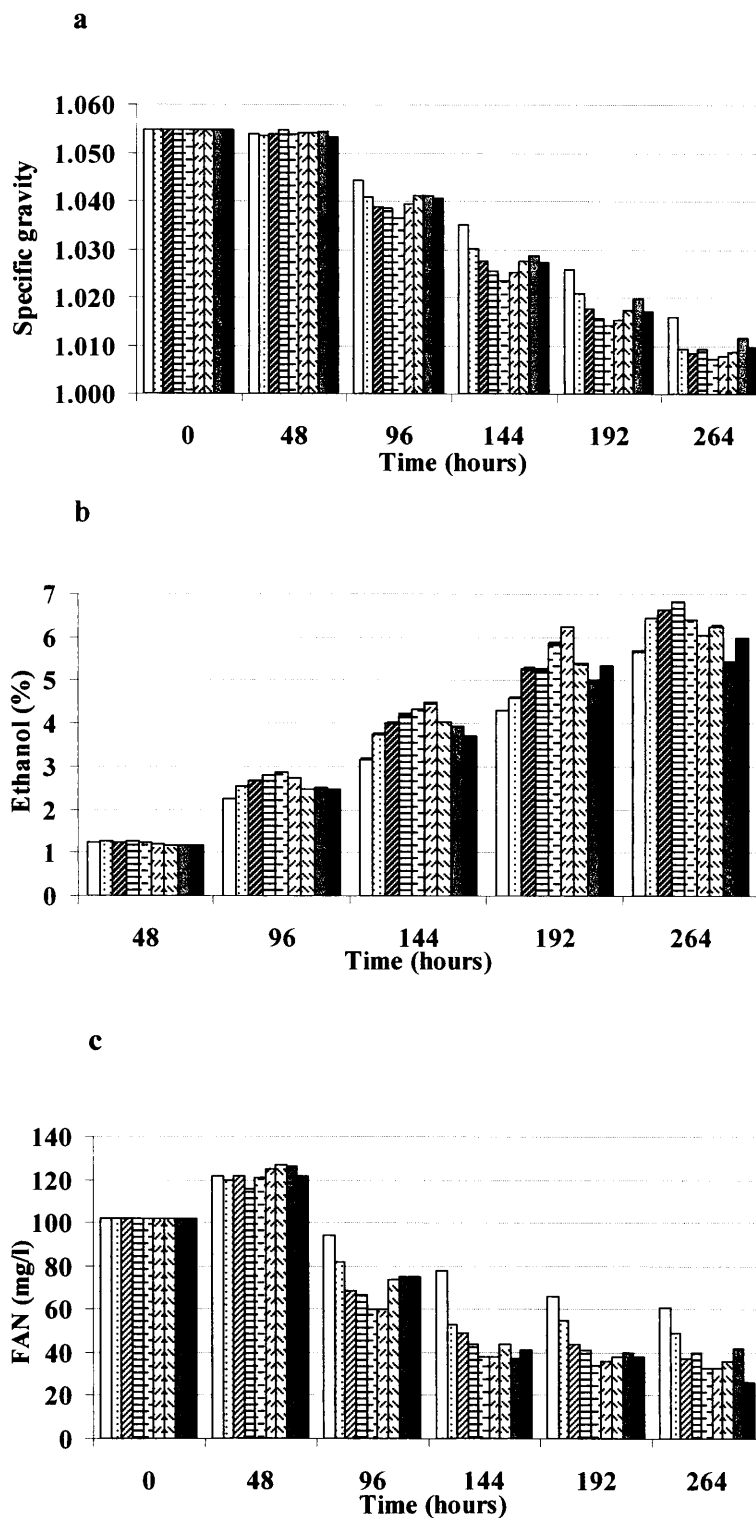


Figure 5.7 Divalent cation levels in wort during fermentation of a brewing yeast strain in 1 L conical vessels. Calcium (a), magnesium (b) and manganese (c) were analysed during fermentation to determine the interactions between zinc and other divalent cations. The symbols indicate the following Zn concentrations (ppm): 0 (□), 0.48 (▤), 1.07 (▥), 4.85 (▧), 10.83 (▨), 23 (▩). Ca and Mn levels constantly decreased during fermentation. Mg wort levels decreased in the first 24 hours to be subsequently re-absorbed by yeast cells (as described by Walker and Maynard, 1997).

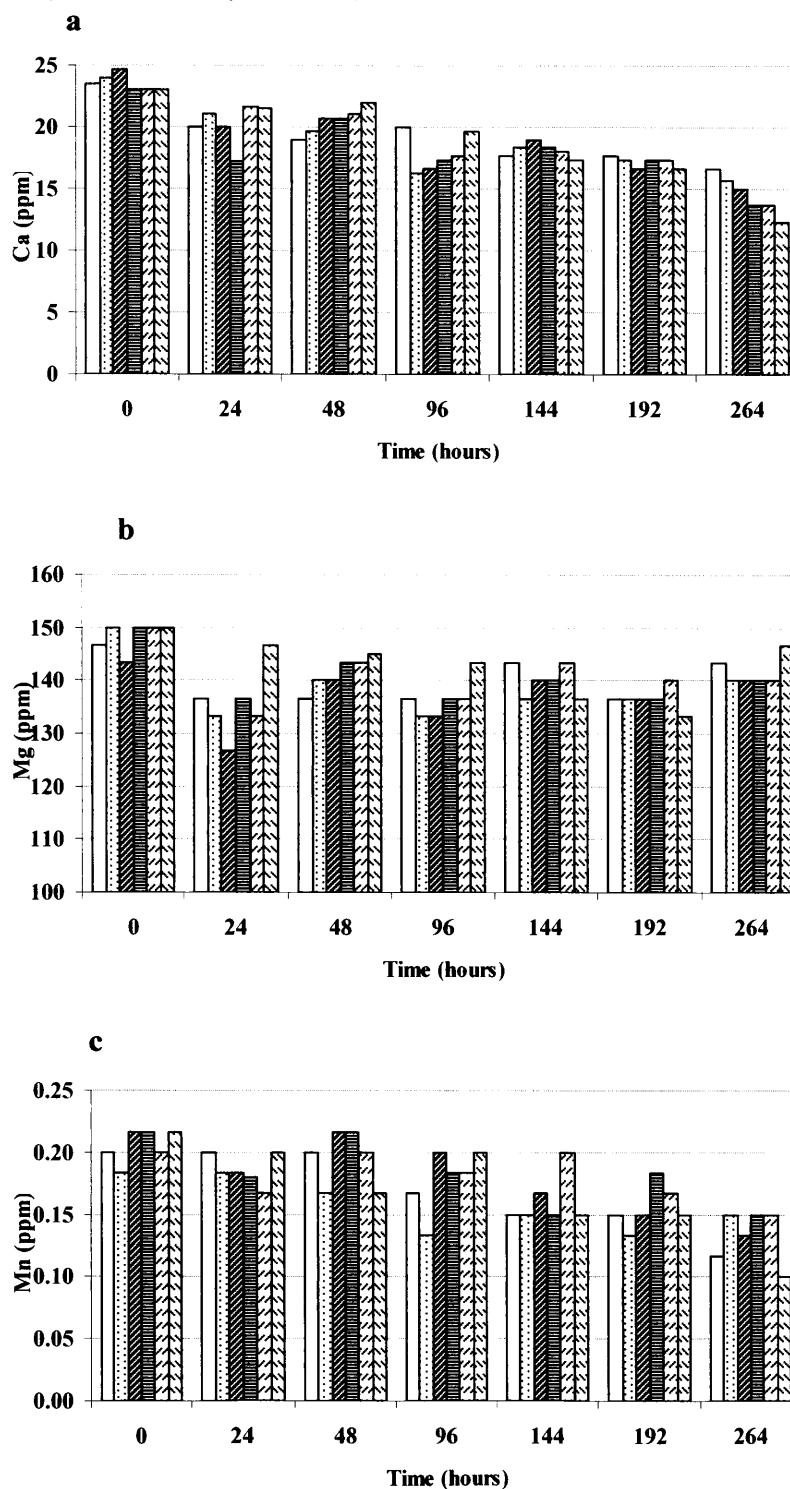
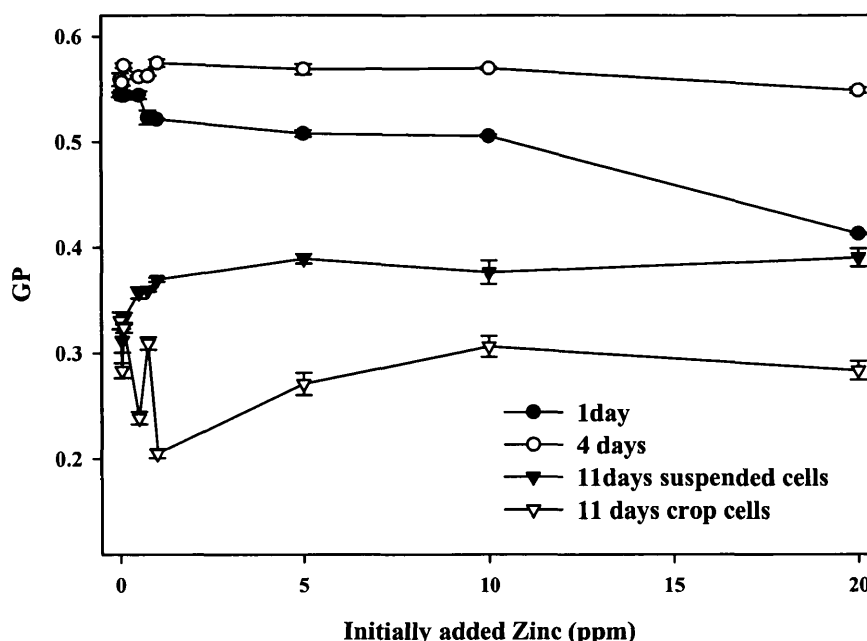


Figure 5.8 Yeast membrane fluidity measured by Generalised Polarisation (GP) at different levels of zinc during fermentation by brewing yeast. Cell GP of the lager brewing strain B under fermentation in conical Imhoff vessels (volume 1L, cone angle 74°) at 14°C. Results represent mean and standard deviation of 3 measurements. Where error bars are not shown they are less than the symbol size.



Studies on influence of zinc on brewing fermentations (pilot plant)

In brewing pilot plant experiments, zinc was quickly accumulated by the yeast cells as previously described in shake flasks or in small scale conical vessel experiments (Fig. 5.9). Zinc cell content was high in the first days of fermentation and gradually decreased (when expressed as zinc/cell) subsequently after cell division. During yeast crop formation, cells were collected from the bottom of the fermenters with 0.5 and 5 ppm of zinc and analysed for intracellular zinc content (Fig. 5.10). Similar levels of intracellular zinc were observed in both suspended and sedimented cells, in medium with 5 ppm of zinc. On the contrary, at 0.5 ppm of extra-cellular zinc, after 138 and 162 hours, zinc content in suspended cells was found respectively 25% and 50% higher compared with cells in the yeast cone. Similarly, Powell *et al.* (2004) found heterogeneity in age and fermentation performance in cells from different yeast crop fractions. Although age (based on image analyses of the bud scars) and fermentation performance were not examined in cells sampled in the present research,

according to this theory, heterogeneity in zinc cell content would also be expected at various fractions of the yeast cone. In small scale brewing experiments, cells of the yeast cone were found to have a lower Generalized Polarization (and therefore higher membrane fluidity) compared with cells in suspension. Although GP levels were not measured in the present experiment, it is conceivable that low intracellular zinc levels in the yeast crop were also associated with altered fluidity of the plasma membrane. As a consequence, zinc may play a role in the stability of the plasma membrane (Binder *et al.*, 2000; Garcia *et al.*, 2005). In chapter 4, it has already been observed that higher zinc cell content can be accumulated by bigger cells (mothers) and this may be trivially related to cellular age. If cell volumes of mother and daughter cells differ at cytokinesis, then the rapid zinc uptake rate would generate heterogeneities in terms of intracellular zinc and other rapidly transported nutrients.

All zinc concentrations examined in the pilot plant study did not dramatically influence the fermentation performance as shown by specific gravity and ethanol production data (Figs. 5.11a; 5.11b). This was probably due to the high initial zinc content of the seed culture (8.18 fg/cell), which was about 8 times higher than in experiments with brewing small-scale conical vessels (1.22 fg/cell). As a consequence, the preconditioning of yeast cells with zinc before inoculation, e.g. in propagation vessels, may be a good way to avoid the direct supplementation of zinc salts into the media during fermentation. Walker *et al.* (1996) and Smith and Walker (2000) already demonstrated that preconditioning cells with magnesium enhanced fermentation performance in terms of rate and yield of ethanol produced. Mg-preconditioned distiller's or brewer's yeasts, with elevated levels of intracellular magnesium, were more active in terms of fermentation performance compared with non-preconditioned cells with normal levels of intracellular Mg. This may be a good solution to occasional nutrient deficiency problems encountered by German brewers who have to respect the "Reinheitsgebot purity law" (see chapter 1, paragraph 1.3.3) and are not allowed to add any other ingredients than yeasts, water, malt and hop during the brewing process.

Analyses of pH (Fig. 5.14) and turbidity (Fig. 5.15) confirmed no particular differences in fermentation performance among various zinc concentrations examined. No differences in

cell number (Fig. 5.12), biomass (Fig. 5.13) and viability (between 90 and 95% at the end of fermentation) were observed and this was in agreement with previous studies in conical vessels and with the work of Jones and Greenfield (1984) and Bromberg *et al.* (1996).

With regard to higher alcohol and ester analyses in the final beer, at the end of fermentation, only at 10 ppm of zinc were some differences found for the following congeners: ethyl caproate, isoamyl acetate, iso-butanol, amyl alcohol, diacetyl and total higher alcohols (Table 5.1). The ethyl caproate and isoamyl acetate are two esters responsible for apple sour and fruity banana aroma, respectively. The synthesis and the release of esters to the media is known to be strain dependent (lager strains more than ale strains) and influenced by several parameters such as wort specific gravity, wort nitrogen content, wort oxygen, wort lipid content, fermentation temperature, the activity of several enzymes and the concentration of acetyl-CoA and the higher (fusel) alcohols (Verstrepen *et al.*, 2003). The esterification of alcohols with acetyl-CoA results in esters. For example, isoamyl acetate is produced by esterification of the amyl alcohol with acetyl-CoA. Quilter *et al.* (2003) observed a continuing increase of amyl alcohol (about 25%) as well as isoamyl acetate in brewing fermentations when zinc was increased from 0.015 to 0.120 ppm. Zinc can stimulate the production of higher alcohols and hence indirectly the production of esters (Hodgson and Moir, 1990; Skanks *et al.*, 1997). Seaton *et al.* (1990) showed that zinc facilitated the breakdown of α -ketoacids to the related higher alcohols. Among the other fusel alcohols stimulated by zinc, iso-butanol and diacetyl are known to impart to beers, respectively, a desirable warming character and a strong “butterscotch” or “toffee” aromas and taste (Briggs *et al.*, 2004). Because reduction of acetaldehyde to ethanol by ADH is zinc-dependent, zinc deficiencies in wort may lead to excess acetaldehyde production. This gives grassy, green-apple taste to beer.

The combination of zinc and EDTA has been proved to slow down staling in beer, helping to prevent oxidation of flavour active compounds and hence producing flavour stability (Rogers *et al.*, 2005). This phenomenon occurs by competition of zinc with other redox active cations, such as Fe and Cu mainly. Zinc binds to the sulphydryl group of thiols in proteins present in beer. Thiols could be involved in a cyclical round of reactions, from free thiols to dithiols, sulphonates returning to thiols. Thiol oxidation can produce peroxide

(Muller, 1997). The competition of zinc and iron for the thiol amino acid interferes with the transfer of two electrons to O₂ (Searle and Tomasi, 1982), resulting in peroxide formation. This can happen only in the presence of the chelator EDTA which reduces the levels of Fe and Cu, competing with zinc. It would be interesting to analyse Fe in beer and to carry out regular sensorial analyses to determine the influence of zinc on the flavour stability during beer aging.

Overall, studies on the influence of micronutrients on yeast fermentation performance are extremely difficult in industrial scale, especially for those metals whose yeast requirements are in the ppb levels. This is due to the practical difficulties to avoid metal contamination from the fermenters in use, as already shown in chapter 7 (Zinc limitation studies in continuous chemostat culture). Only acid wash treatments of the fermentation vessels would ensure metal ion removal. Acid treatment was technically not possible on a large scale and some zinc may have been released from the vessels to wort during the fermentation process. The large volume of culture required as inoculum for the fermenters and the resulting need to use cells coming from production (from the storage vessels, after one fermentation production) has not allowed the precise control of the zinc levels in cells used as seed culture. The brewery where the experiment was conducted appeared to have practiced satisfactory zinc supplementations during propagation and the previous fermentations. Therefore relatively high zinc levels in pitching yeast resulted in optimal fermentation performance at all zinc concentrations studied.

Figure 5.9 Zinc cell content of a brewing yeast strain during fermentation in 200 L pilot plant fermenters with variable zinc concentrations. A lager brewing strain (lager B) was inoculated in 200 L fermenters. Fermentation was carried out for 8 days in malt wort where Zn levels were altered up to 10 ppm with zinc sulphate. Cells in suspension were sampled from a port placed in the middle of the fermenter (see also Fig. 2.3). A sample of 5 L was collected after a pre-wash of the port. Cells were harvested after centrifugation and washed thrice with dH₂O (see chapter 2 Materials and Methods) prior to acid digestion and Zn analyses by AAS.

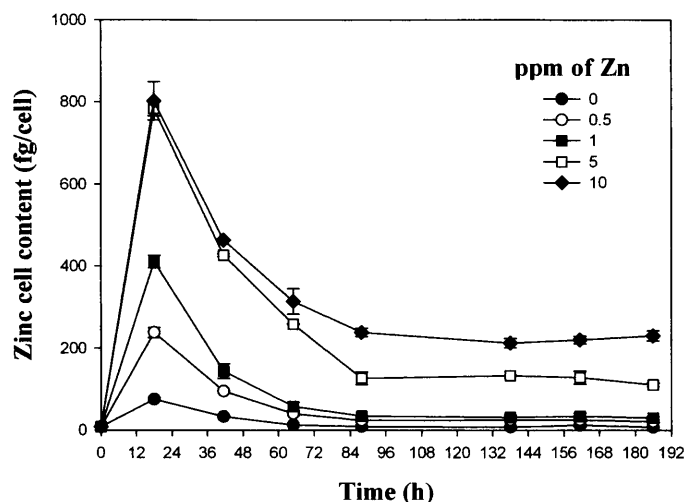


Figure 5.10 Zinc content of suspended and precipitated cells of brewing strain at the end of fermentation in 200 L pilot plant fermenters. Cells in suspension and in the yeast cone were sampled from fermenters with zinc levels at 0.5 and 5 ppm to compare the zinc cell content. After 138 and 162 hours, sedimented yeast in the crop contained respectively 25% and 50% lower intracellular zinc concentration than yeast in suspension in the 0.5ppm fermentation.

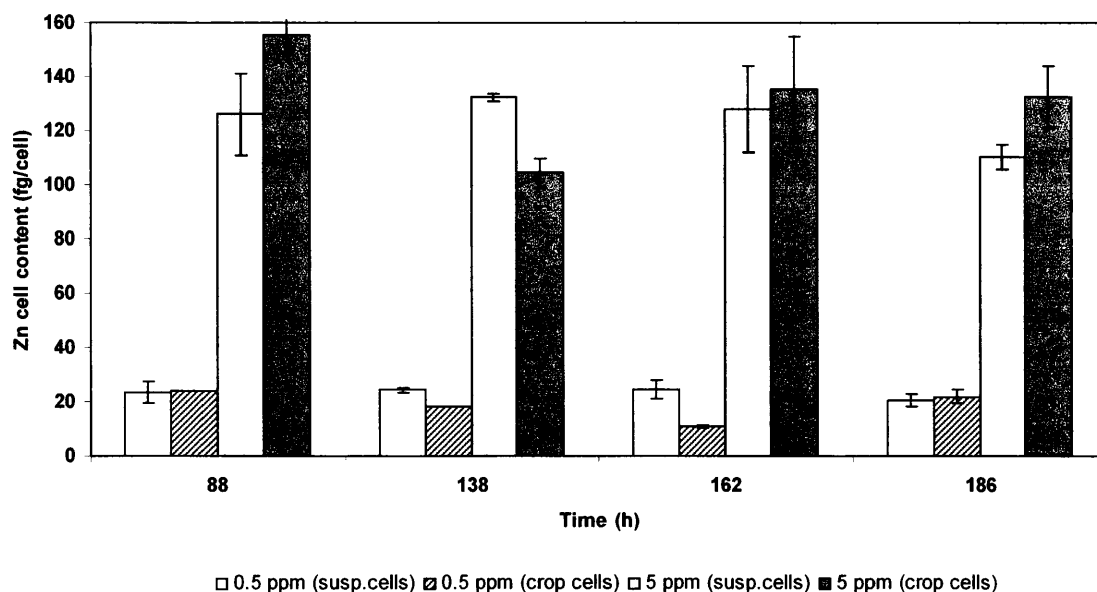


Figure 5.11 Influence of zinc on wort gravity and ethanol levels during fermentation of a brewing yeast strain in 200 L pilot plant fermenters. Specific gravity (a) and ethanol (b) analyses were carried out during fermentation to determine the fermentation rate at various zinc levels.

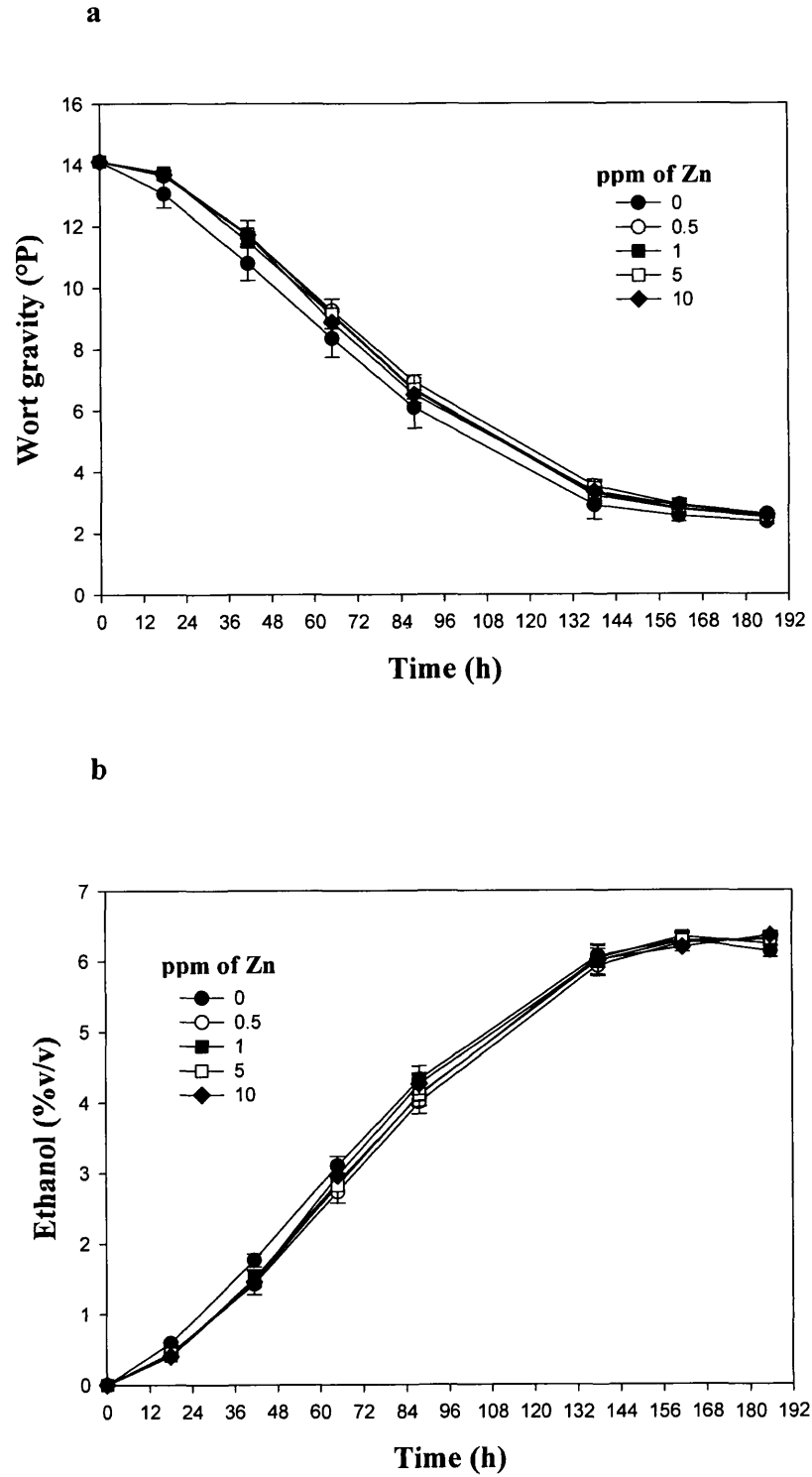


Figure 5.12 Influence of zinc on growth of a brewing yeast strain during fermentation in 200 L pilot plant fermenters. Cells in suspension were analysed by Counting Coulter to determine cellular growth and flocculation levels at various zinc concentrations

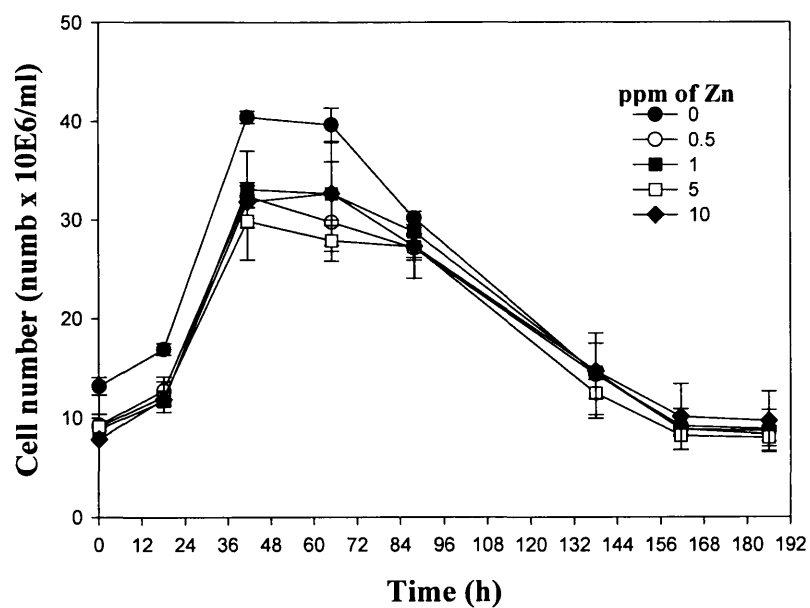


Figure 5.13 Influence of zinc on biomass of a brewing yeast strain during fermentation in 200 L pilot plant fermenters. Cellular growth was measured by dry weight determination.

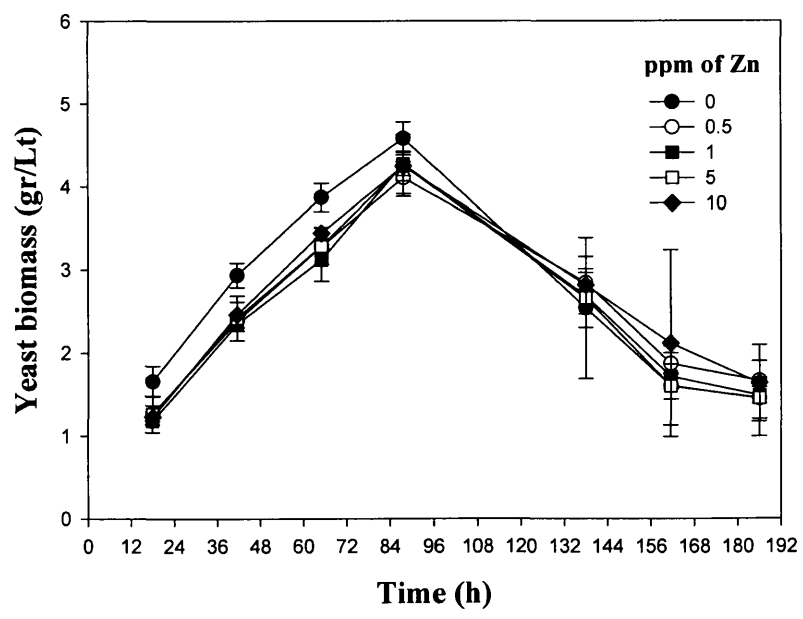


Figure 5.14 Influence of zinc on pH during fermentation of a brewing yeast strain in 200 L pilot plant fermenters.

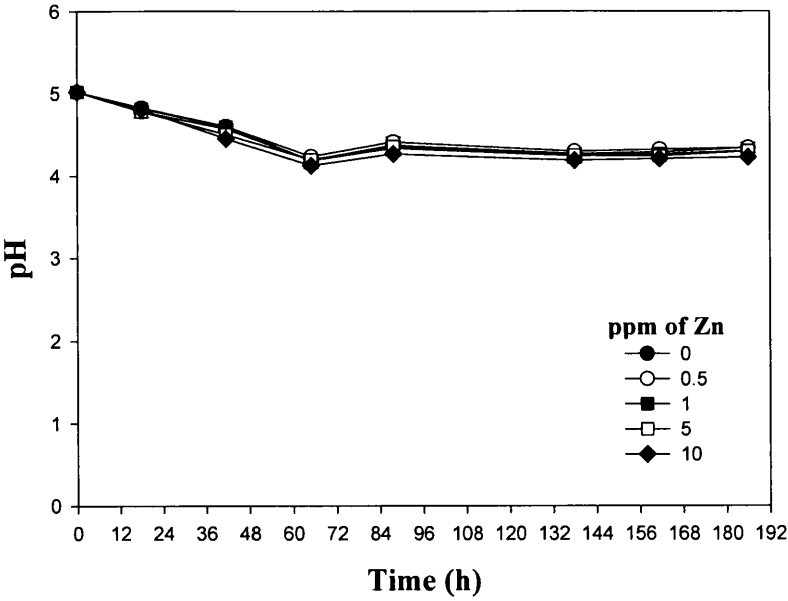


Figure 5.15 Influence of zinc on wort turbidity during fermentation of a brewing yeast strain in 200 L pilot plant fermenters.

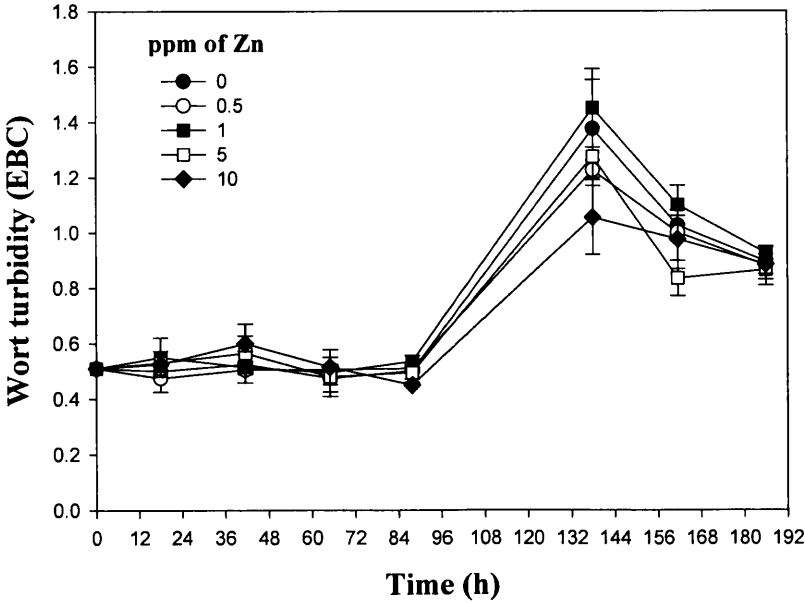


Table 5.1 Influence of zinc on esters and high alcohols in green beer obtained from the pilot plant experiment after 8 days of fermentation.

		Initial zinc in wort				
		0 ppm	0.5 ppm	1 ppm	5 ppm	10 ppm
Acetaldehyde (ethanal)	mg/L	7.25	10.70	8.70	9.20	8.05
Dimethylsulphide (DMS)	µg/L	31.90	32.15	32.30	32.40	32.70
Acetone (propanone)	mg/L	0.20	0.20	0.21	0.20	0.19
Ethylformiate	mg/L	0.10	0.10	0.10	0.10	0.10
Ethylacetate	mg/L	31.00	27.30	30.85	29.05	32.50
Methanol	mg/L	2.00	2.01	2.18	2.10	2.01
Ethyl propionate	mg/L	0.15	0.10	0.10	0.10	0.10
n-Propylalcohol (1-propanol)	mg/L	13.86	12.65	12.79	12.63	13.67
Iso-butylalcohol (iso-butanol)	mg/L	19.50	19.35	19.60	20.20	21.20
Iso-amylacetate	mg/L	4.09	3.72	4.54	4.27	5.10
Amyl alcohols	mg/L	81.00	77.65	79.55	79.50	83.65
Ethyl caproate (ethylhexanoate)	mg/L	0.20	0.20	0.20	0.20	0.25
Total higher alcohols	mg/L	114.35	109.70	111.95	112.35	118.50
Diacetyl (2,3-butanedione)	µg/L	94.20	90.70	100.65	99.75	114.65
2,3-Pentanedione	µg/L	128.70	132.70	126.60	127.75	122.15

Studies on the influence of zinc on wine fermentations (small scale conical vessels)

Zinc uptake patterns by the wine yeast strains studied (Figs. 5.16.a; 5.16b) were different from those described in the shake flask experiments (Fig. 3.5). Although zinc uptake was high in the first two days of fermentation, wine strains A and B tended to release zinc back into the medium after, respectively, 48 (Fig. 5.16.a) and 24 (Fig. 5.16b) hours. This behaviour may have depended on the strains in use. Most likely when ethanol levels were above 6%, (Figs. 5.20.a; 5.20.b) yeast cells continuously exposed to such a relatively high concentration may have had altered plasma membrane permeability, resulting in loss of zinc ions (see also chapter 6 on the “Influence of physical and chemical stresses on yeast zinc homeostasis”). In this regard, Learmonth and Gratton (2002) reported that ethanol stress increased the membrane fluidity of the yeast cells.

Yeast growth of the two wine strains was not dramatically affected by the zinc concentrations of this study (Figs. 5.17.a; 5.17.b). At the end of the fermentation, the yeast cone formed at the bottom of the fermenters was approximately of the same size in all the fermenters. Only the wine strain A growing in medium with 0.9 ppm of zinc, had a slower growth rate and lower final biomass. Zinc concentrations of 2.5 ppm and 2.9 ppm respectively affected wine strains A and B by accelerating the flocculation process of the yeast cells and giving a slightly higher final biomass. The viability of both strains was not affected by any of the zinc concentrations tested and at the end of the fermentation was 84% for the wine strain A and 83% for the wine strain B.

Although zinc cell contents of yeast remained homogenous at different levels of the yeast cone (data not shown), for wine yeast A, a cell size gradient was apparent in the cone (i.e. larger cells at bottom, smaller cells on top) (Fig. 5.19a). The yeast cone of the wine strain B was heterogeneous with regard to yeast size (Fig. 5.19b). This data has not practical relevance since in wine-making industry, yeast biomass is not recycled for several fermentations. Contrarily, this result can be compared with preliminary experiments on the influence of zinc in brewing small scale conical vessels (see paragraph “Studies on zinc influence on brewing fermentations, small scale conical vessels”), where homogeneity in cell size was found within the yeast cone. The hypothesis was that the small size (1L) of the conical vessels may have influenced the distribution of the yeast cells. The behaviour of the wine strain A may have depended on the flocculation properties of this particular strain.

Various zinc concentrations did not dramatically affect the fermentation rate. Wine strain A showed higher production of ethanol at 2.5 ppm of zinc (Fig. 5.20a). This result, together with a higher growth rate, appeared to indicate that this concentration had a general beneficial effect on this strain. The wine strain B did not show the same pattern. The optimal zinc concentration was certainly strain dependent. Moreover zinc requirements for best fermentative performance appeared higher in wine yeast strains (e.g. 2.5 ppm) compared with lager strains (0.4-1 ppm).

Overall, in the wine fermentation experiments it was not possible to have grape juice with zinc concentration below 0.9 ppm and a yeast seed culture depleted in zinc. This may have contributed to reduce the differences between various zinc levels as discussed for the brewing fermentation in pilot plant.

Figure 5.16 Zinc supernatant levels during fermentation of wine yeast strains in 1 L conical vessels with variable zinc concentrations Yeast cells of the strain Wine A and Wine B were inoculated in Imhoff conical vessels, in grape juice with zinc levels adjusted adding Zn acetate. Fermentation was carried out at 25°C for 120 hours (5 days). Zn residual levels in supernatants were analysed throughout fermentation for both strains Wine A (a) and Wine B (b).

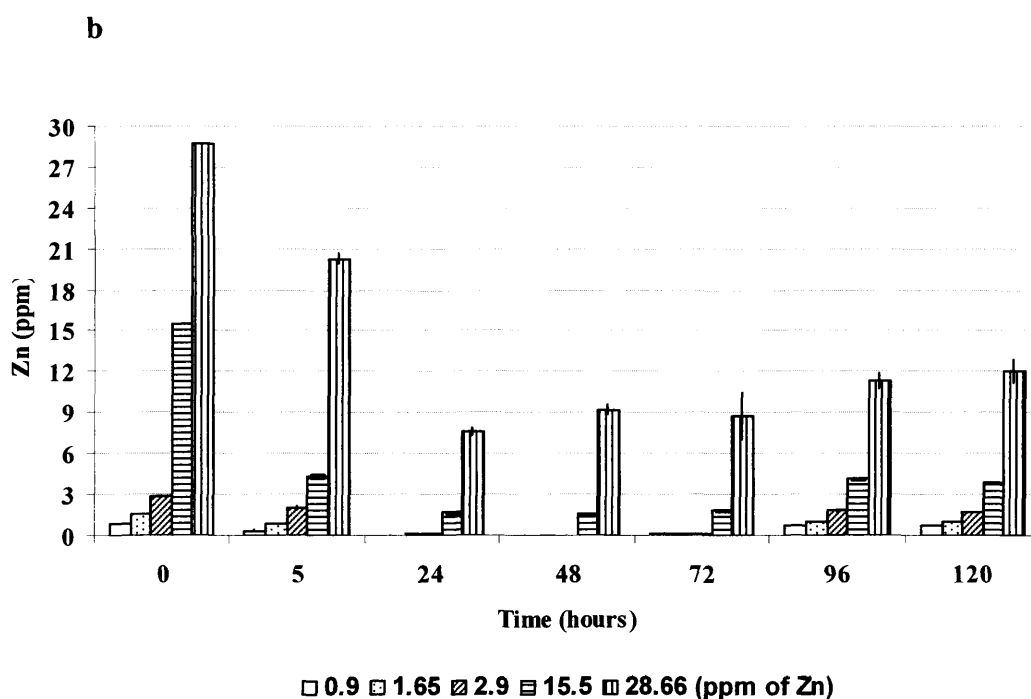
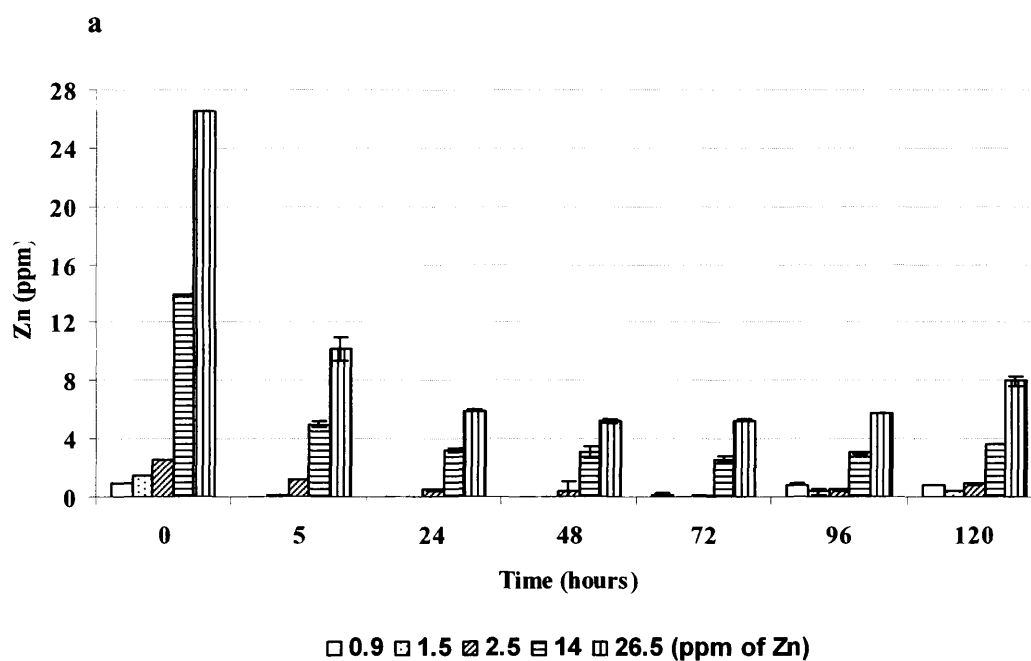


Figure 5.17 Influence of zinc on growth of the wine yeast strain A during fermentation in 1 L conical vessels. Cell growth was checked daily considering both the yeast cone formed at the bottom of the conical vessels by sedimentation of the cells (a), and the cell number of cells in suspension (b).

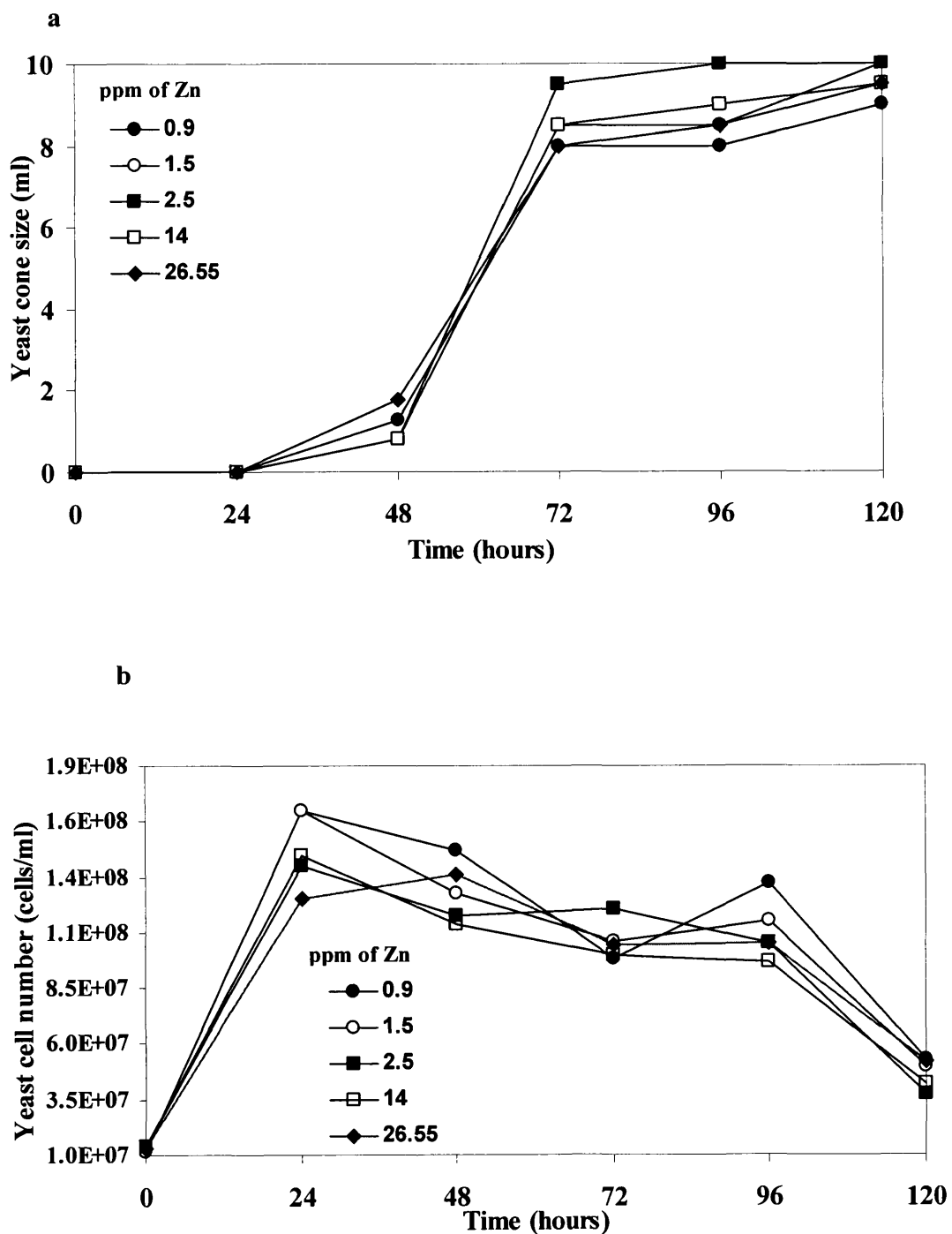


Figure 5.18 Influence of zinc on growth of the wine yeast strain B during fermentation in 1 L conical vessels. Cell growth was checked daily considering both the yeast cone formed at the bottom of the conical vessels by sedimentation of the cells (a), and the cell number of cells in suspension (b).

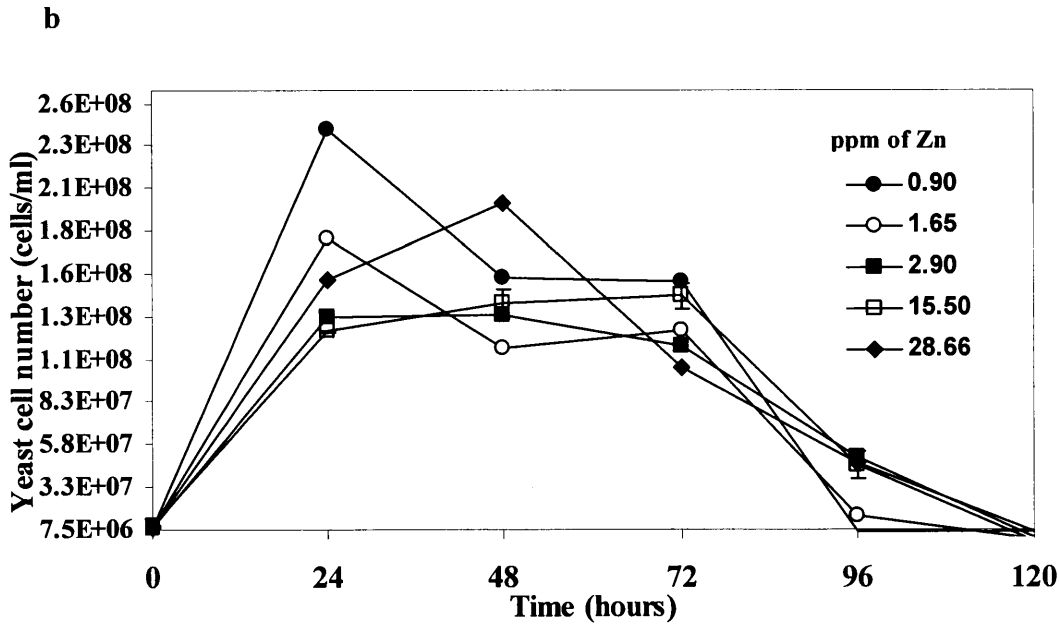
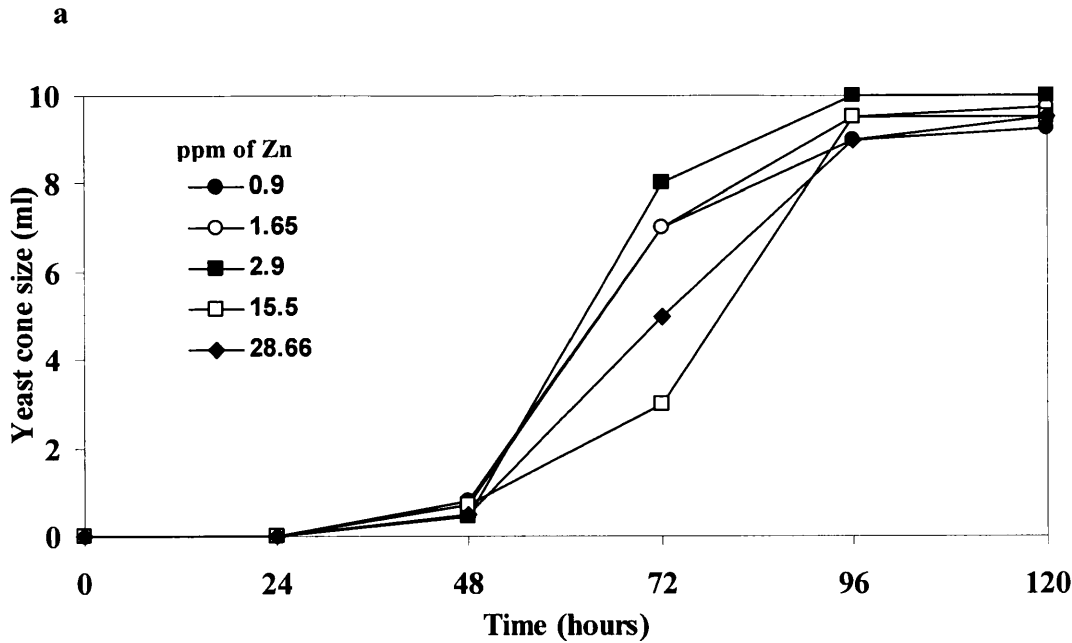


Figure 5.19 Influence of zinc on yeast cell size at the end of fermentation. At the end of the fermentation, cell samples of the wine strain A (a) and wine strain B (b) were taken from the top, bottom and middle of the yeast cone using syringes with long needles. The size of the cells was analysed by Coulter Multisizer.

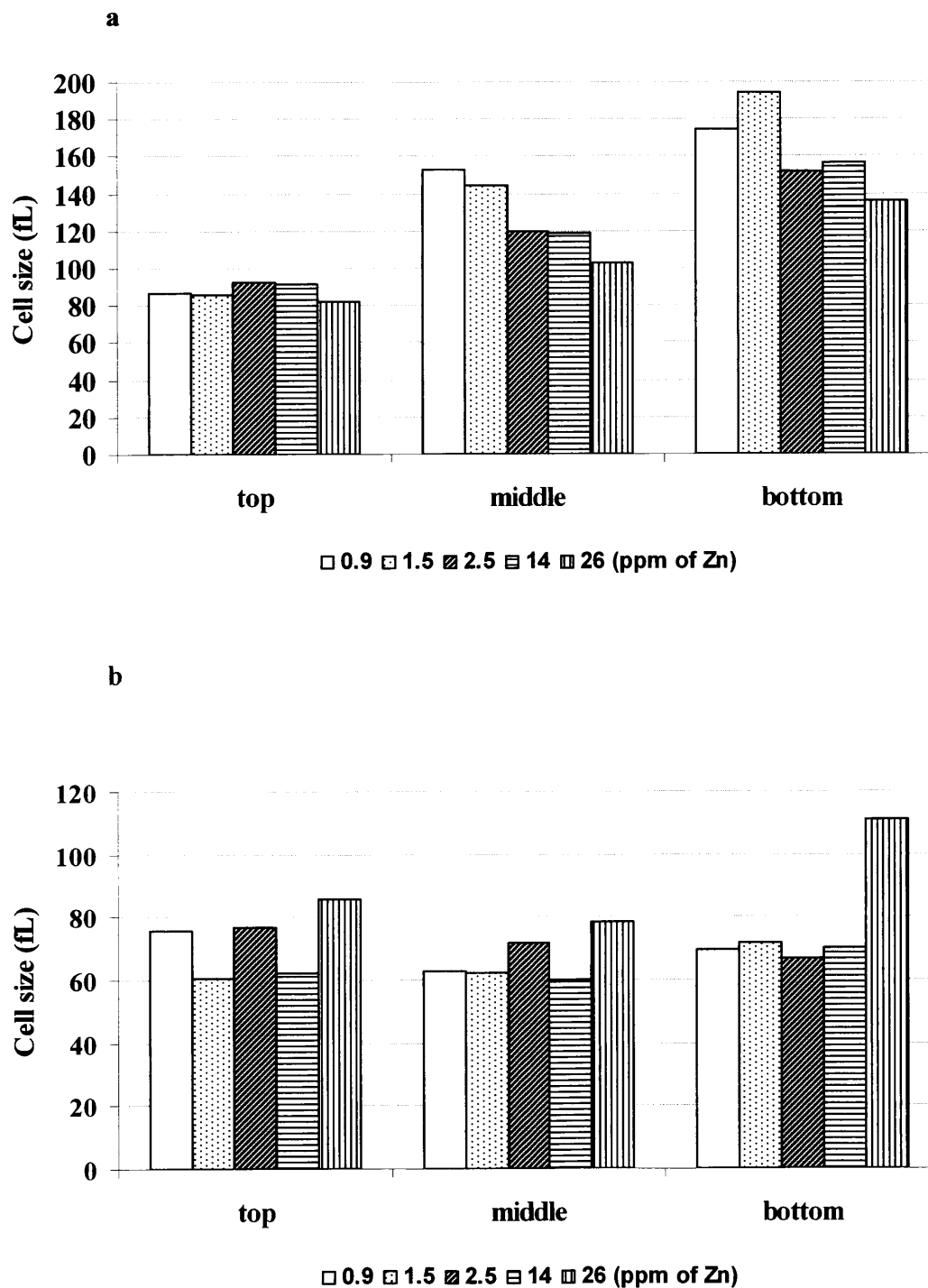
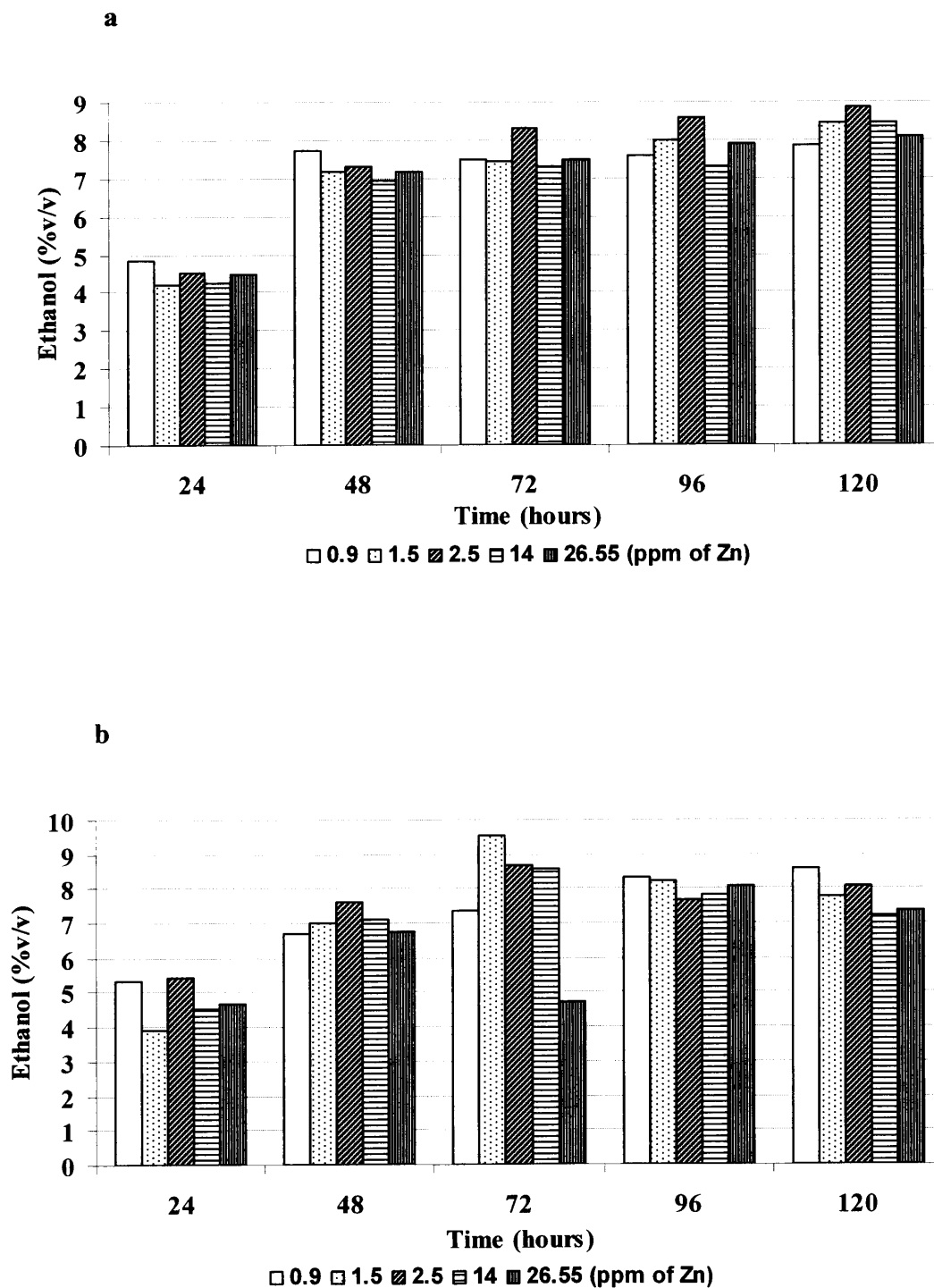


Figure 5.20 Influence of zinc on ethanol levels during fermentation of wine strains in 1 L conical vessels
 Fermentation performance of the wine strain A (a) and wine strain B (b) was evaluated by analysing the ethanol produced daily



5.4 Summary

The results of the research presented in this chapter showed that:

- Zinc concentration in the range 0.4- 1 ppm gave fastest fermentation rates in brewing fermentation and using lager strain B. At lower concentrations, fermentations were sluggish. High levels of zinc (eg. 23 ppm) were not toxic to yeast, but did slow fermentation rates. The optimal zinc level was yeast strain dependent.
- Various zinc concentrations in the range 0-23 ppm did not adversely affect yeast cell viability in both brewing and wine yeasts.
- Irrespective of zinc availability, yeast membrane fluidity varied with culture age. There was a complex relationship between yeast cell zinc status and membrane fluidity, but it appeared that cells with low zinc content exhibited low GP levels indicating high membrane fluidity. Such cells would be more susceptible to membrane fluidisation by ethanol.
- Brewing yeast cells preconditioned with zinc performed very well, even in very low zinc wort.
- Initial zinc levels influenced esters and higher alcohol profiles in green beer.
- At pilot plant brewing scale, the intracellular zinc content of yeast may be different at various fractions of the yeast cone. Attention should be paid to selectively remove appropriate parts of the yeast cone when the yeast cells are recycled for the following fermentation.
- Some yeast strains may release zinc back into the medium. This phenomenon was observed in the wine strains and may depend on the ethanol stress imposed on cells during the fermentation process.

Chapter 6

Influence of physical and chemical stresses on zinc homeostasis in brewing yeast

6.1 Introduction

The physico-chemical properties of the environment can strongly influence the physiology of yeast cells, affecting both viability and vitality. In the alcohol industry, at any stage of the process, yeast cells can encounter a variety of stresses which can have a significant impact on cell growth and fermentation performance. Yeasts respond to stresses by changing their metabolic activities and by adapting their physiology in order to protect the cellular components from damage, to survive and to recover when optimal environmental conditions are re-established. The understanding and the correct management of the interactions between environmental stresses and yeasts is fundamental for brewers, wine-makers and distillers who want to optimise cell growth, viability and fermentation performance.

In industrial processes, the stresses most commonly encountered are chemical (e.g. ethanol and other metabolite toxicity, oxidative, anaerobiosis, pH changes and acid wash treatments, nutrient limitation/starvation, metal ion toxicity/limitation), physical (e.g. osmostress, changes in temperatures, dehydration, rehydration, mechanical shear, hydrostatic pressure) and biological (e.g. cell aging, genotypic changes or competition from other organisms) (Walker, 1998). Table 6.1 reports a list of the stresses that yeast cells have to face during the brewing process and the physiological responses to these insults. Ethanol and temperature are the most common stresses in the brewing process and they will be dealt with in more details in this introduction.

Table 6.1 Stresses encountered by yeasts in the brewing process (adapted from Walker and Van Dijck, 2006).

Stress	Stage of operation	Yeast responses
	Stock culture preservation	
Freeze-drying		Trehalose synthesis
Ultrafreezing		Differential gene expression
Cryopreservation		Antifreeze protein induction
	Inoculum preparation and biomass propagation	
Osmostress		Glycerol synthesis
Temperature shock		Hsp induction
Oxidative stress		Antioxidant production
Nutrient limitation (including metals)		Altered gene expression
Osmostress-heat shock		Hsp induction
Nutrient excess (including metals)		Glycerol synthesis
	Fermentation	
Declining pH, nutrient limitation (including metals)		Altered membrane structure
High ethanol, CO ₂ ,		Trehalose synthesis
Other toxic compounds (acetaldehyde, acids, etc.),		CytP450
no sugars		Glycogen accumulation
	Post –fermentation (yeast harvesting, storage, acid washing)	
Mechanical shear		Protease release
Cold shock		Trehalose synthesis
Nutrient starvation (including metals)		Induction of cold shock proteins
pH shock		
Anaerobiosis		

Ethanol is normally produced by yeasts during the fermentation process. *S. cerevisiae* is known to be particularly tolerant to ethanol compared with other yeast species. Some strains of *S. cerevisiae* are able to tolerate ethanol concentrations up to 20% without major effects on growth and metabolic activity (Walker and Van Dijck, 2006) and for this reason have been selected for fermentation processes aimed at producing fuel ethanol (bioethanol). In malt wort with increasing osmotic pressure of the media, during the early stages of the fermentation (after 3 hours), ale strain cells were found to accumulate intracellular ethanol concentration at higher levels compared with extra-cellular levels. During the remaining period of fermentation, intracellular and extracellular ethanol concentrations were similar (D'Amore *et al.*, 1988a). Except the early stages, normally, ethanol transport from yeast cells to the external medium occurs by free diffusion and no ethanol is accumulated intracellularly (Guijarro and Lagunas, 1984) (see also table 1.2, chapter 1).

Ethanol toxicity inhibits glycolytic enzymes (Ingram and Bukkte, 1984). Increasing concentrations of ethanol initially inhibit yeast cell metabolism and latterly lead to cell death (D'Amore *et al.*, 1988a; Walker, 1998). Ethanol impairs growth rates, cell division and viability (Walker, 1998). Yeast cells exposed to critical concentrations of ethanol change their morphology, cell size, cell wall shape and roughness. These changes depend on both the ethanol concentration and the exposure time to the stress and are more evident in *Schizosaccharomyces pombe* than in *S. cerevisiae* (Canetta *et al.*, 2006). Due to the ethanol solvent action, high ethanol concentrations immediately increase yeast membrane fluidity except in cells in early growth rate, where the initial increase in fluidity is followed by a marked sustained decrease (Learmonth and Gratton, 2002). The denaturation of membrane proteins may be the primary reason for such a phenomenon. Under ethanol stress, a decrease in unsaturated lipids of the yeast cell membrane and a reduction of the membrane sterol content occurs. Plasma-membrane ATPase activity is stimulated by ethanol, as well as with increasing temperatures, and contributes to proton extrusion and increased ionic permeability (Piper, 1995). The loss of the plasma membrane permeability barrier due to the disruption of the membrane structure integrity, can eventually lead to cellular death (Marza *et al.*, 2002). Trehalose accumulation, induction of the Hsp30 membrane protein and the heat shock proteins (Hsp70 and Hsp90) are some yeast responses to ethanol stress which are also common to heat stresses (Piper, 1995).

Studies on global gene expression in conditions of ethanol stress are still in the early stages with a large number of identified genes over-expressed during this chemical stress (Alexandre *et al.*, 2001; Rossignol *et al.*, 2003). Signalling molecules involved in the communication of environmental changes to the interior of the cell have been recently discovered. For example, two proteins Asr1p (Betz *et al.*, 2004) and Rat8p (Takemura *et al.*, 2004), that usually shuttle between nucleus and cytoplasm, were found to be localised reversibly near the nucleus during ethanol stress, but not under other environmental stresses, demonstrating that some cellular response differences exist between cells stressed with heat and ethanol.

Although the brewing fermentation process is carried out in anaerobic conditions, yeast cells are exposed to oxidative stress during propagation and at start of pitching. The oxygen generates the so called reactive oxygen species (ROS) such as superoxide anions (O_2^-),

hydrogen peroxide (H_2O_2) and the hydroxyl radicals (OH^\cdot), both in the growth medium or into the yeast cell. These free radicals can cause cellular damage to DNA, proteins and lipids. This may lead to physiological problems and impaired fermentation performance. Yeast cells respond to these compounds by generating enzymes such superoxide dismutase (Mn Sod and Cu/Zn Sod), catalases (peroxisomal catalase A and cytoplasmic catalase T), (Lushchak and Gospodaryov, 2005) and peroxidases. Non-enzymatic antioxidants such as glutathione and metallothioneins may be also synthesised to, respectively, scavenge oxygen radicals or detoxify metal ions, protecting cells from oxidative damage (Walker, 2006). The tolerance to oxidative stress is strain dependent and related to the growth phase (Martin *et al.*, 2000).

In the brewing and bioethanol industry, after fermentation and prior to yeast storage or pitching, yeast cells may be washed with bactericidal substances in order to decrease the level of contaminating bacteria. Several bacteria, both Gram+ (Priest, 1999) and Gram- (Van Vuuren, 1999) are known to cause beer spoilage, unwanted flavours and aromas or cloudy beer. Since the use of antibiotics is banned in brewing and alcohol production, with the only exception of the Brazilian bioethanol industry (J. McLaren, personal communication), acids such as phosphoric acid, ammonium persulphate or a mixture of ammonium persulfate with phosphoric acid (Cunningham and Stewart, 1998) can be employed to decrease the pH to about 2-2.5. The low pH and the residual hop acids bound to the yeast cell walls, have a synergistic detrimental effect even on the more resistant bacteria (e.g. lactic bacteria). The plasma membrane ATPase activity, which generates the electrochemical gradient (see also paragraph 1.2.1, chapter 1) for ion transport, is regulated by external and intracellular pH, with optimum pH functional levels between 6.0 and 6.7 (Jones and Gadd, 1990). Other factors influencing the ATPase activity are the internal levels of ATP and ADP, the cellular Mg^{2+} (as required cofactor) or Ca^{2+} (as inhibitor). Yeast cells can usually deal with very low pH but in conditions of starvation, high cell density and low temperatures (5°C), their physiology can be negatively affected and consequently also the fermentation performance.

High temperatures (above 40°C) have several effects on the cell physiology and morphology of non-thermotolerant yeasts. For example, cell viability decreases, cell

surface shrinks and cell volume reduces (Adya *et al.*, 2006), plasma membrane fluidity increases affecting nutrient permeability (Learmonth and Gratton, 2002), cell growth is inhibited with cells stopping their cycle at G1 phase and both respiration and fermentation are inhibited (Walker and Van Dijck, 2006). Such consequences may be more pronounced depending on the yeast strain, the exposure time to the stress and the temperature levels (Adya *et al.*, 2006). Yeast cells respond to temperature stresses through various mechanisms of defence. Heat shock proteins (Hsp), such as Hsp70 and Hsp90 involved also in the ethanol stress, are synthesised to act as chaperones, in order to prevent protein aggregation, to act as antioxidants, to denature damaged proteins and to protect and translocate *de novo* proteins to their subcellular location (Morano *et al.*, 1998). A primary role in thermo-stress protection has been given to the protein Hsp104 (Lindquist and Kim, 1996). When *S. cerevisiae* was gradually pre-adapted (from 25 to 50°C at 0.5°C/min) to the heat stress of 50°C for 1 hour, cells were able to survive 50-fold higher than the rapidly heated cells, even if they were treated with cycloheximide, a well known protein synthesis inhibitor. Once the temperature was cooled down to 25°C, the thermotolerance mechanism was inhibited. Since Hsp104 was not synthesised, other mechanisms, probably involving the plasma membrane, appeared to be implicated during this heat stress (Guyot *et al.*, 2005). Intracellular trehalose accumulation is another response to heat stress (but also oxidative and osmotic stress) required to stabilise cell membranes. Trehalose is normally synthesised by yeast cells from glucose 6-phosphate and is accumulated in large amounts when cells are grown in glucose, in late exponential phase and at beginning of the stationary phase. When glucose is limiting, trehalose is usually accumulated when half of the glucose is consumed (Briggs, 2004). The synthesis of trehalose during temperature stress is due to its capacity to stabilise cell membranes, by binding to both the inner and outer surfaces of the phospholipids (Eleutherio *et al.*, 1993). Low temperatures are also known to affect yeast physiology and morphology. At 4-10°C, cell division arrests (Walker and Van Dijck, 2006) and at 0°C for 1 hour, cell surface shrinks maintaining their original cell size and viability (Adya *et al.*, 2006).

Temperature stresses are often encountered in brewing. Although the optimal growth temperature for brewing strains is between 30 and 34°C, ale fermentations are usually performed at 18-22°C, higher compared with lager temperatures (8-15°C). If the temperature is higher than the suggested ranges, the attenuation time can be reduced but

off-flavours and higher alcohols or esters, particularly undesirable in lager beers, can arise influencing beer aroma (Briggs, 2004). Heat is produced during the fermentation process due to the metabolism of wort sugars. The energy is stored intracellularly as ATP (see also chapter 1, paragraph 1.2.3) and then it is dissipated during fermentation with the maximum peak at maximum fermentation rate. A cooling system is therefore required to decrease the fermenter temperature and keep the temperature conditions optimal for beer fermentation. Yeast cells can also be insulted by cold stresses, to which cells respond with the synthesis of trehalose. During yeast storage, prior to pitching, nutrient starvation, high cell density, oxygenation and low temperatures can cause a combined stress for yeast cells.

In brewing, master cultures can be preserved by cryopreservation in liquid nitrogen (-196°C), by deep-freezing ($-80^{\circ}\text{C}/-130^{\circ}\text{C}$) or freeze-drying. The cryopreservation of yeasts is a technique employed for the long term maintenance to ensure genetic stability of the yeast cultures. Many cryoprotectant additives are known to protect *S. cerevisiae* from the mechanical and physiological damages due to freezing and thawing processes, e.g. dimethylsulfoxide (Me_2SO), glycerol (also in combination with 5% lactose or skimmed milk, raffinose or maltose), ethanol, propylene glycol, sorbitol (2M), trehalose and bacteriological peptone (10%) (Hubalek, 2003).

Freeze-drying consists in the rapid dehydration of frozen yeast biomass by sublimation of the water using vacuum. Anhydrobiosis (life without water) is a complex phenomenon of events aimed at maintaining the native structure of the molecules and preventing oxidative damage. The aim of a good preservation is to keep the cytoplasm in a stable glassy state in order to reduce the diffusion of molecules and to decrease the chemical reactions. During freeze-drying, the water is rapidly lost from the cytoplasm, while some is transferred from the vacuole to the cytosol to keep minimal metabolic functions and to protect cellular components (e.g. proteins, DNA, membrane lipids) from oxidative stress (Espindola, 2003). For this purpose, both enzymatic (see above) and non enzymatic compounds are synthesised (e.g. glutathione, glycogen and trehalose).

In brewing, yeast cells may face osmotic stress at the moment of pitching into fresh malt wort. This insult is often encountered in modern breweries where high gravity malt worts (e.g. 18°P , 1060 OG) are used (Cunningham and Stewart, 1998). Due to the recent

increasing demand of beer in certain countries, high gravity malt worts have become essential for those breweries wanting to increase beer production without enlarging brewing facilities. High gravity worts can impose a hypertonic stress due to higher osmolarity of the external medium (wort) compared with the osmolarity of the intracellular fluids (White *et al.*, 2003). This condition produces efflux of intracellular water, loss of cell viability and shrinkage and reduction of the cellular volume, depending on the extra-cellular concentrations and the exposure time to the stress (Adya *et al.*, 2006). Yeast cells respond to osmotic stress by increasing the cytoplasmic concentrations of osmolytes such as trehalose and glycerol. These solutes can bind molecules of water thus recalling water from the media and retaining intra-cellular water. At the same time, they do not influence enzyme activity, protein denaturation, membrane degradation and general cellular metabolism. In high gravity worts, accumulation of trehalose was found to be higher than that of glycerol from the very early stages of the fermentation (Majara *et al.*, 1996).

Generally speaking, the mechanisms described above and activated by yeast cells during temperature stresses are similar to those stimulated by ethanol, osmotic and oxidative stresses. In brewing, the stresses illustrated above can often occur simultaneously. During the brewing process, brewers (and distillers) can intervene in various ways to protect cells from these insults and to optimise the fermentation process. For example, the supplementation of minerals has been proved to be beneficial to yeast cell viability. The effect of magnesium in protecting yeast cells from ethanol and high temperature stresses has been described by Birch and Walker (2000). Most probably, magnesium has a role in plasma membrane stability (Hu *et al.*, 2003a) and Hu *et al.*, (2003b) ascribed the same role also to calcium. The anti-oxidant properties of zinc in biological systems have already been reviewed (Berg and Shi, 1996; Truong-Tran *et al.*, 2001) (see also paragraph 1.4.2). The assumption is that zinc may also have protective functions on cellular compartments, enzymes and more generally on the metabolic activities of the yeast cells.

6.2 Experimental

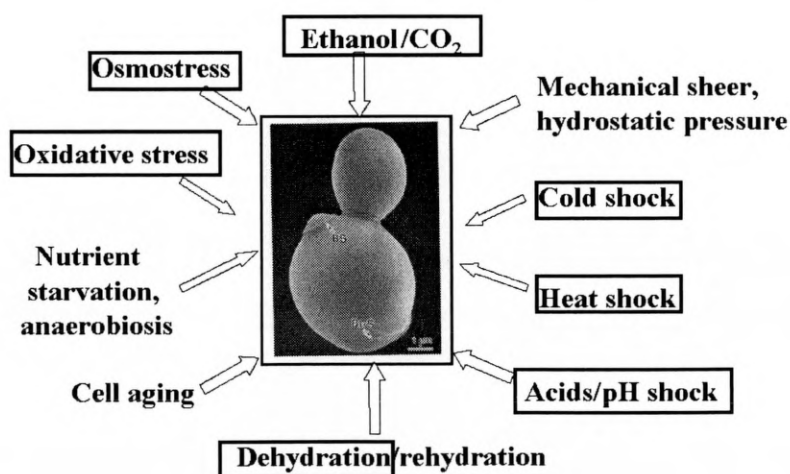
Experimental aims

The effect of a selection of industrial stresses, normally encountered in the brewing process was investigated on the zinc cellular content of a lager brewing strain. The role of high zinc intracellular levels on cell protection and of the plasma membrane fluidity on zinc homeostasis were also examined during these insults. Stress due to zinc-limitation will be dealt with in detail in chapter 7.

Experimental approach

Yeast cells of the lager strain B were cultured in malt wort with normal (0.7-1.13 ppm) or high levels (22-38 ppm) of zinc and insulted with the following individual stresses (Fig.6.1): ethanol, oxidative, osmotic, temperature, dehydration, cryopreservation. A combination of other stresses (high cellular density with carbon dioxide) was also investigated to simulate conditions encountered in the brewing process during yeast storage. Zinc cellular content and viability were analysed during exposure to these stresses. Magnesium and ATP intracellular levels were also measured during ethanol and temperature stresses in order to determine the role of the plasma membrane on zinc homeostasis.

Figure 6.1 Yeast cell stresses related to the brewing process studied in this Thesis. The most common stresses encountered in the brewing process are represented in this figure. Stresses highlighted by frames were investigated in this Thesis.



Experimental procedure

Seed culture of the lager strain B in malt wort, in shake flask at 25°C, 180 rpm, for 24 hours

↓

Inoculum of 5×10^6 cells/ml in two shake flasks, in malt wort (1060 OG) at 25°C, 180 rpm, for 24 hours. One flask was unsupplemented with zinc (Zn at 0.7-1.13 ppm). The second flask was supplemented with zinc acetate to have a concentration in the range 22-38 ppm of Zn.

↓

Cells were collected after 24 hours, washed once with dH₂O and resuspended in deionised shake flasks, keeping the same cell density as the culture, excepted the acid-washing and combined stress experiments (experimental conditions depended on the type of stress).

↓

Sampling (at 1, 3, 5, and 24 hours)
for: cell number, viability, zinc cell content

Notes:

- In ethanol stress experiments, ethanol concentrations tested were: 0, 2.5, 5, 10 and 20% (v/v), for 24 hours.
- In oxidative stress experiments, yeast cells were resuspended in solutions with hydrogen peroxide (H₂O₂) at the following concentrations: 0, 0.01%, 0.1% and 1% (v/v), for 24 hours.
- In acid-washing experiments, cells were resuspended at 2.3×10^8 cells/ml in dH₂O (control), dH₂O acidified to pH 2.2 with 3M phosphoric and 0.75%(w/v) ammonium persulfate acidified to pH 2.2 with 3M phosphoric acid. Suspensions were kept on ice with mild agitation for 2 hours to simulate the process usually employed in breweries to control bacterial contaminants in pitching yeast.
- In temperature stress experiments, cells were resuspended in dH₂O and stressed at the following temperatures: 0, 8, 30, 45 and 65°C, for 24 hours.
- In the cryo-stress experiments, cell suspensions in 20% glycerol were stored at -20°C for 2 hours, at -80°C for 24 hours and transferred in liquid nitrogen (-196 °C) where they were kept for 14 days prior to analyses. Microscopic images were captured using Atomic Force Microscopy (AFM). See paragraph 2.9, chapter 2.

- In osmostress experiments, cells were resuspended in sorbitol solution at the following concentrations: 0, 2.5%, 5%, 10%, 20% (w/v), for 24 hours.
- In freeze-drying experiments, cells were washed once in dH₂O and concentrated 16 times prior to quick freezing in liquid nitrogen and transferring to a freeze-dryer Micro Modulyo (from Edwards) connected to a vacuum pump for 24 hours. Microscopic images were captured using Atomic Force Microscopy.
- In stress experiments where it was required to prepare chemical solutions (hydrogen peroxide, ethanol and sorbitol), they were checked for zinc concentrations. The solutions were found to contain no zinc. All glassware was acid-washed to remove any traces of zinc (see procedure in Materials and methods).
- Cellular ATP and magnesium were analysed during heat stress at 45°C, 20% ethanol and a combination of the two stresses, for 24 hours. ATP was analysed using the method described in paragraph 2.5.1, chapter 2.
- In the brewery-simulated stress, yeast cells were grown for 48 hours in malt extract broth (Zn at 0.25 ppm), washed in dH₂O and resuspended in dH₂O (control) or in lager beer (from Heineken, Zn at 0 ppm, alcohol 5%). Cells were resuspended at high cell density (4.5×10^8 cells/ml) and maintained at 0°C for 2 hours in Buchner flasks. A vacuum pump was used to extract the O₂ and prior to sealage of the flasks. These procedures were required to simulate the industrial storage of the yeast slurry, where high yeast cell densities are kept starved, at low ethanol levels (~5%), at low temperature and high CO₂ levels.

6.3 Results and discussion

Zinc and ethanol stress

Yeast cells stressed in ethanol were able to maintain high viability and retain original intracellular levels of zinc within the 24 hours of stress (Fig. 6.2), regardless of their initial zinc cell content. The only exception was the highest ethanol concentration tested (20%v/v) that produced a high loss of viability: 8.8% in normal and 0% in high zinc cell content (Figs. 6.2a; 6.2c) after 24 hours. High intracellular zinc (70 fg/cell) did not protect cells from ethanol stress as it was demonstrated for magnesium (Birch and Walker, 2000; Hu *et al.*, 2003a) or calcium (Hu *et al.*, 2003b). Although initially, high cellular zinc content did not influence cell viability, it is conceivable that zinc ions might have replaced sites normally assigned to other elements, normally known to interact with zinc (Mg, Ca, Fe, Mn) (see also paragraph 1.5.4, chapter 1). This hypothesis does not exclude that more suitable cellular zinc contents may also determine an increased resistance to cell mortality. Since zinc and viability loss were coincident, intracellular mechanisms related to cellular death were possibly determining zinc release. Influence of ethanol on membrane permeability has already been observed by Learmonth and Gratton (2003). In that study, the authors used the probe Laurdan to determine the Generalised Polarization (GP) parameter, as an index of cellular membrane fluidity. It is plausible that highest membrane fluidities arising at high ethanol concentrations influenced the permeability of the tonoplast, the vacuolar membrane. Since most of the zinc is stored in the vacuole when accumulated in large amounts (see figure 4.3, chapter 4 and MacDiarmid *et al.*, 2003), changes in fluidity of the tonoplast may have determined zinc release. This theory is also supported by recent studies on the influence of zinc on phospholipid synthesis (Iwasnyshyn *et al.*, 2004; Han *et al.*, 2005; Carman, 2005). Zinc depletion was found to highly affect the *DPPI* gene encoding DGPP phosphatase (Lyons *et al.*, 2000) an enzyme associated to the vacuole membrane and involved in the synthesis of phosphatidate and diacylglycerol. Lack of zinc reduced these two compounds and phosphatidylserine, increasing the production of phosphatidylinositol (Han *et al.*, 2005). In general, the mechanisms by which zinc influences phospholipid synthesis appear to be complex and associated not only with the *DPPI* gene.

Zinc and oxidative stress

Experiments conducted under oxidative stress did not show any change in terms of viability and zinc cellular content (Fig. 6.3), regardless of the initial zinc content of the cells. The antioxidant effects of zinc (Truong-Tran, 2001) were not appreciable in this study. Resistance to oxidative stress has been shown to be dependent on the growth phase (with stationary-phase populations more resistant than exponential-phase cells), the medium and the strain, with lager strains more tolerant than ale strains (Martin *et al.*, 2003). Probably the initial zinc content of the stressed cells was satisfactory to face the oxidative stress imposed during these experiments. During aerobic stress, zinc is known to play a key role in one of the metallo-enzymes superoxide dismutase (Cu,Zn-Sod) whose function is to scavenge the potential harmful superoxide anion (O_2^-). Studies on keratinocytes exposed to oxidative stress *in vitro*, with reduced zinc levels, showed that the SOD activities (both controlled by zinc/copper or manganese) were unchanged (Parat *et al.*, 1997). Moreover, zinc limitation studies of a haploid laboratory strain (see chapter 7) showed that higher extra-cellular zinc level was required for cell growth in aerobic conditions compared with anaerobic conditions. Microarray analyses (courtesy of Ms. Hazelwood, see chapter 7) carried out on steady state yeast cells from these experiments, clearly showed that the genes encoding for SOD were not up-regulated. Most likely, SOD activities are not influenced by zinc deficiency.

Zinc and acidic stress

After acid-washing treatments (Fig. 6.4), no major differences were found in viability and zinc content of the stressed cells of the lager strain B. In the short exposure time of the stress (2 hours), zinc cellular homeostasis may have depended on the correct functioning of the proton-pumping ATPase, probably unaffected by the pH decrease, from 4.5 of the growth culture to 2.2 of the acidic stress. Studies on the proton-pumping activity reported that the plasma membrane ATPase increased its activity 2-3 fold when yeast cells were grown in a medium with a final pH of 4 (Jones and Gadd, 1990). For this reason, pH changes were expected to alter zinc homeostasis. Probably, in the experimental conditions of this Thesis, the starvation and the lack of energy sources did not influence the functioning of the plasma membrane ATPase. Mineral acids (e.g. phosphoric), unlike organic acids like acetate, lactate, may not affect intracellular pH which would probably

activate ATPase to pump out protons in an effort to maintain cell pH constant. Further exposure to the stress may have affected both viability and zinc cellular content. Moreover in this study, yeast cells were washed from any residual medium prior to acid stress. The presence of ethanol may have additionally influenced the plasma membrane fluidity (Learmonth and Gratton, 2003). Oxidative stress during acid-washing, as well as yeast storage, was investigated by Martin *et al.* (2003) who did not find evidence of such a stress on yeast cells (catalase activity, glutathione concentrations, glycogen and trehalose levels remained constant). Zinc supplementations during acid-washing treatments were found not to influence yeast fermentation performance (Taidi *et al.*, 2000) and the implications of these findings have already been discussed in paragraph 3.5, chapter 3.

Figure 6. 2 Zinc cell content and viability of brewing yeast under ethanol stress. Cells of the lager strain B were cultured in malt wort with 0.7 ppm (a, b) and 22 ppm zinc (c, d). Cells were harvested, washed once and resuspended in the following ethanol concentrations: 0 (□), 2.5% (▤), 5% (▨), 10% (▩) and 20% (◻) (v/v). Zinc cell contents (a, c) and viability (b, d) were determined during the first 5 hours and after 24 hours.

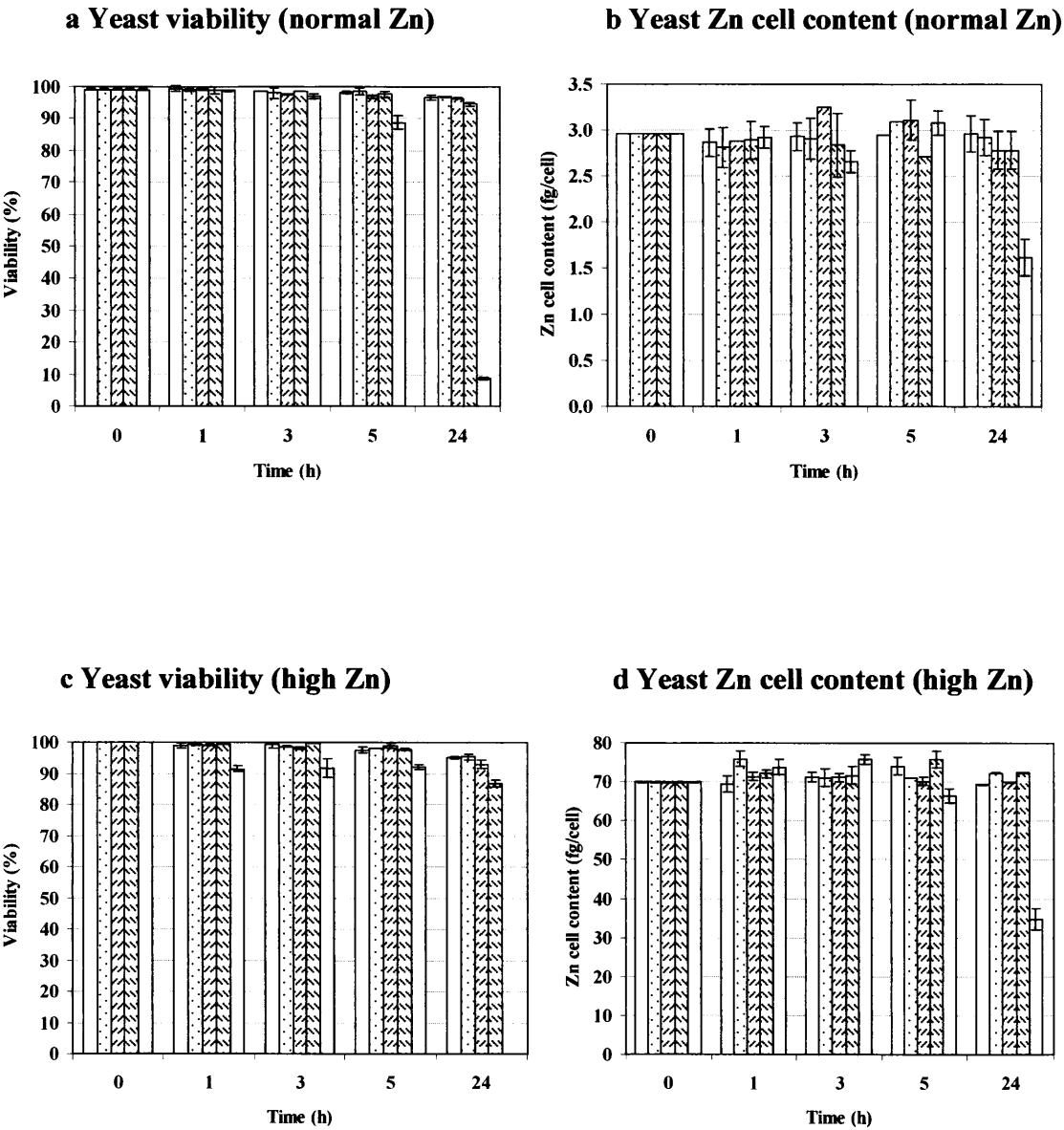


Figure 6.3 Zinc cell content and viability of brewing yeast under oxidative stress. Cells of the lager strain B were cultured in malt wort with 1.03 ppm (a, b) and 25.33 ppm zinc (c, d). Cells were harvested, washed once and resuspended in the following hydrogen peroxide concentrations: 0(□), 0.01%(▨), 0.1%(▩), and 1%(▧) (v/v). Zinc cell contents (a, c) and viability (b, d) were checked in the first 5 hours and after 24 hours.

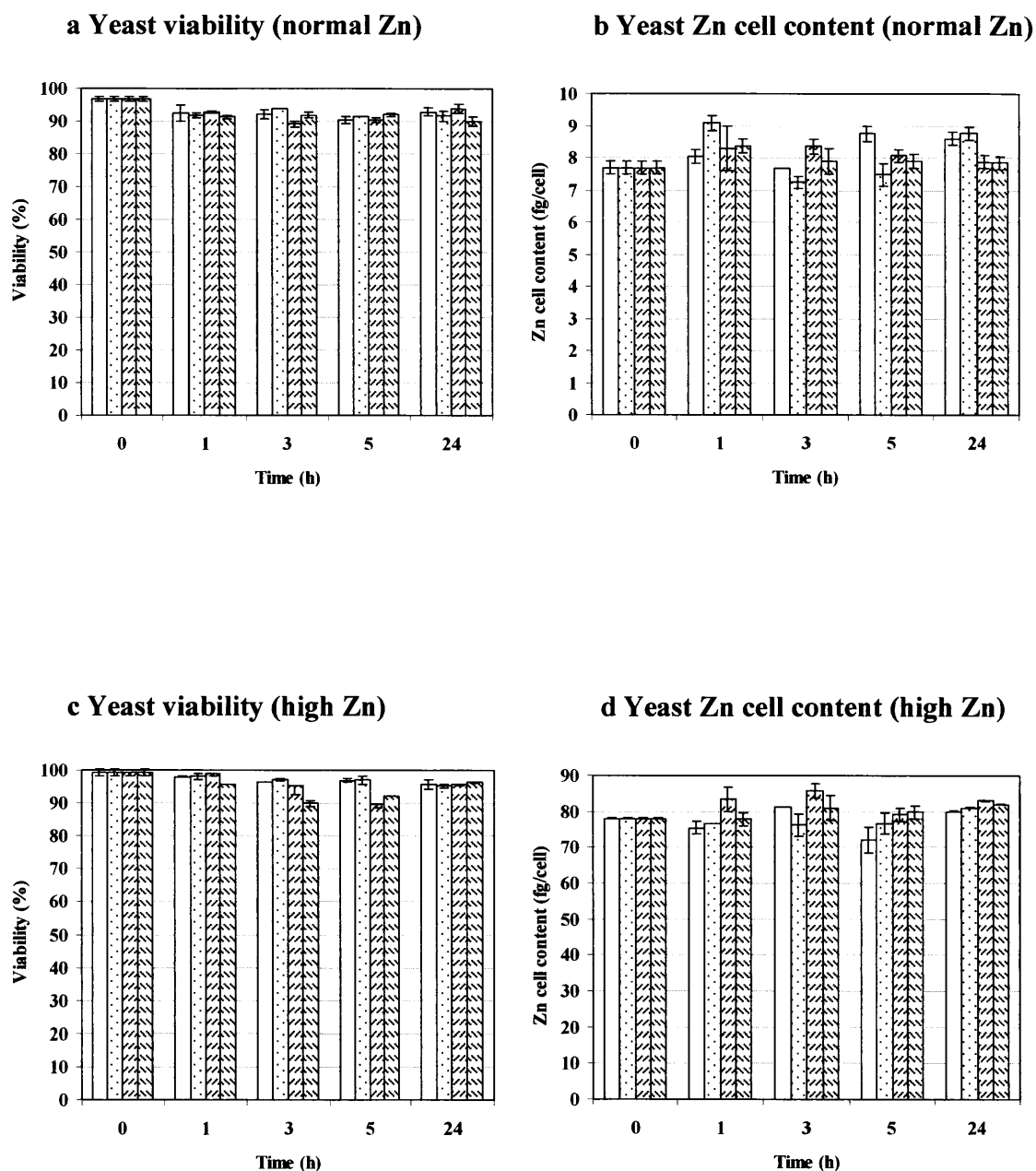
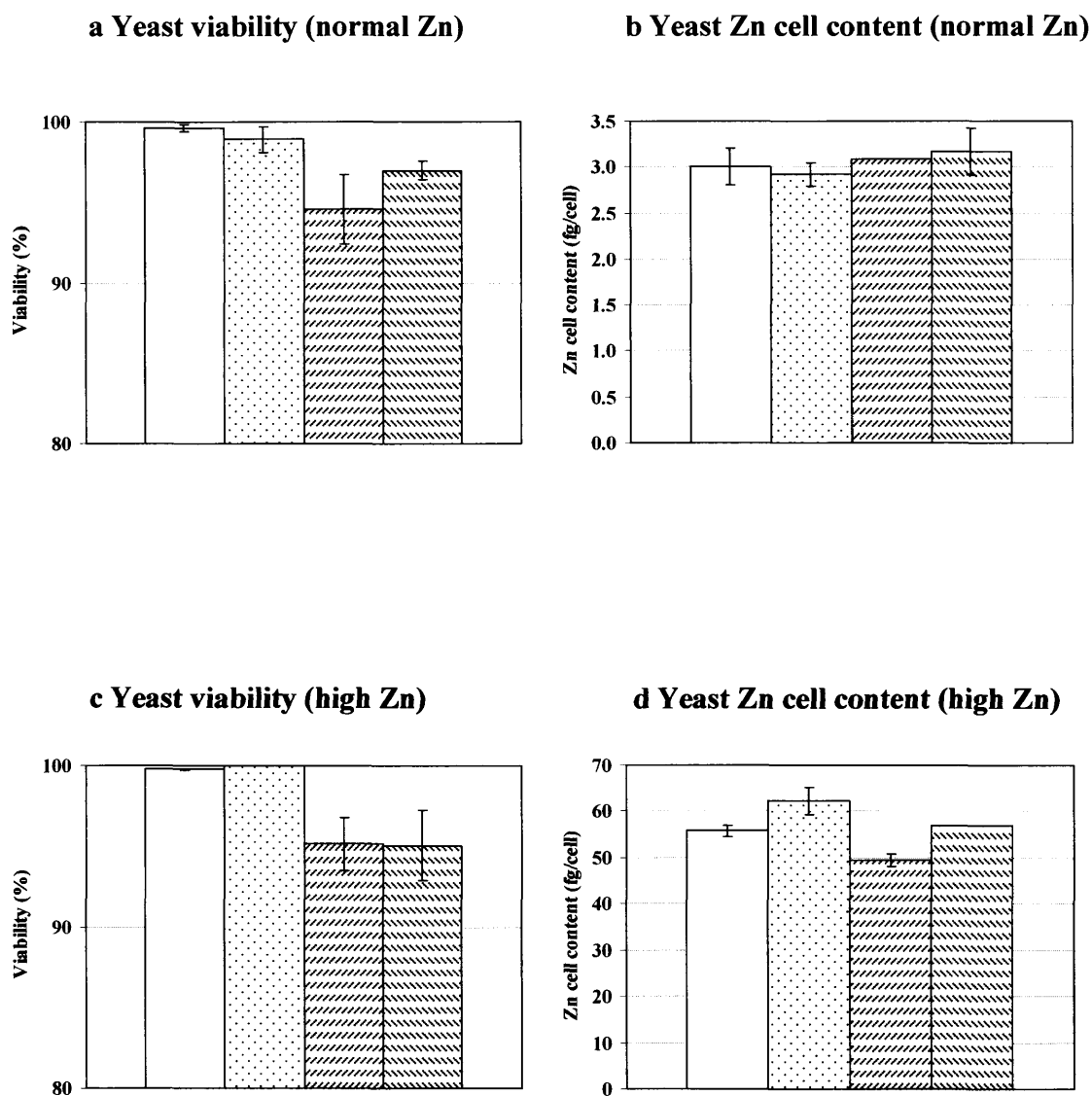


Figure 6.4 Zinc cell content and viability of brewing yeast after acid-washing. Cells of the lager strain B were cultured in malt wort with 0.75 ppm (a, b) and 24 ppm zinc (c, d). Cells were harvested, washed once and resuspended in dH₂O (□) or in solutions of 3M phosphoric acid (▨), at pH 2.2 and 0.75%(w/v) ammonium persulfate acidified to pH 2.2 with 3M phosphoric acid (▩). Seed culture viability and zinc cell content are also shown (□). Zinc cell contents (a, c) and viability (b, d) were checked in the first 5 hours and after 24 hours.



Zinc and temperature stress

When the lager strain B was stressed at various temperatures, the decrease in viability was related to both the increase of temperature and the period of exposure to the stress (Fig. 6.5) as found by Adya *et al.* (2006). Cells were killed within 1 hour at 65°C and within 24 hours at 45°C. At 0, 8 and 30°C, viability was not altered after 24 hours. At the most severe stresses, yeast cells lost most of their intracellular zinc with some residual zinc remaining after 24 hours. The patterns were similar for cells preconditioned in normal zinc (Zn at 4.63 fg/cell) and high zinc (Zn at 123 fg/cell). As for ethanol, temperature is known to influence the plasma membrane by increasing its fluidity (Learmonth and Gratton, 2002). The yeast plasma membrane has been recently suggested to be the primary cellular compartment controlling heat stress tolerance (Guyot *et al.*, 2005). Therefore, it appeared to be the principal fraction responsible for zinc loss. The cellular residual zinc detected at the end of the stress was probably tightly bound to the yeast cell wall. Similarities between heat and ethanol cellular response, in terms of viability and zinc release, were certainly predictable considering that the mechanisms of general cellular response to these insults (e.g. trehalose and induction of the heat shock proteins) have already been described (Piper, 1995).

Zinc and osmotic stress

Osmotic stress produced the same decrease in viability and zinc cell content as described for ethanol and temperature stresses (Fig. 6.6). During osmotic stress, yeast cells lose water, decrease cell volume and shrink with the plasma membrane remaining in close contact to the cell wall. In optimal conditions, membrane phospholipids are usually hydrated and in liquid crystalline phase but under conditions of dehydration, lipids enter into gel phase (Crowe *et al.*, 1984). As a result, cellular membrane functional integrity can be dramatically impaired. Survival to these changes depends on the synthesis of trehalose during dehydration or its degradation during rehydration. Trehalose can replace water and the dry lipids can have similar properties of hydrated lipids. The capacity to metabolise trehalose is related to the growth phase of the yeast cells, with cells in stationary phase accumulating high trehalose levels (Walker, 1998). In this study, cells of the lager strain B were exposed to high levels of sorbitol very quickly and may not have had the necessary time to metabolise trehalose and glycerol, necessary to retain intracellular water. Most likely, this situation produced irreversible damage to the cellular membranes resulting in loss of viability and cellular death.

Figure 6.5 Zinc cell content and viability of brewing yeast under temperature stress. Cells of the lager strain B were cultured in malt wort with 0.97 ppm (a, b) and 38.17 ppm zinc (c, d). Cells were harvested, washed once and resuspended in dH₂O at the following temperatures: 0 °C (□), 8°C (▤), 30°C (▨), 45°C(▩). and 65°C (□). Zinc cell contents (a, c) and viability (b, d) were checked in the first 5 hours and after 24 hours.

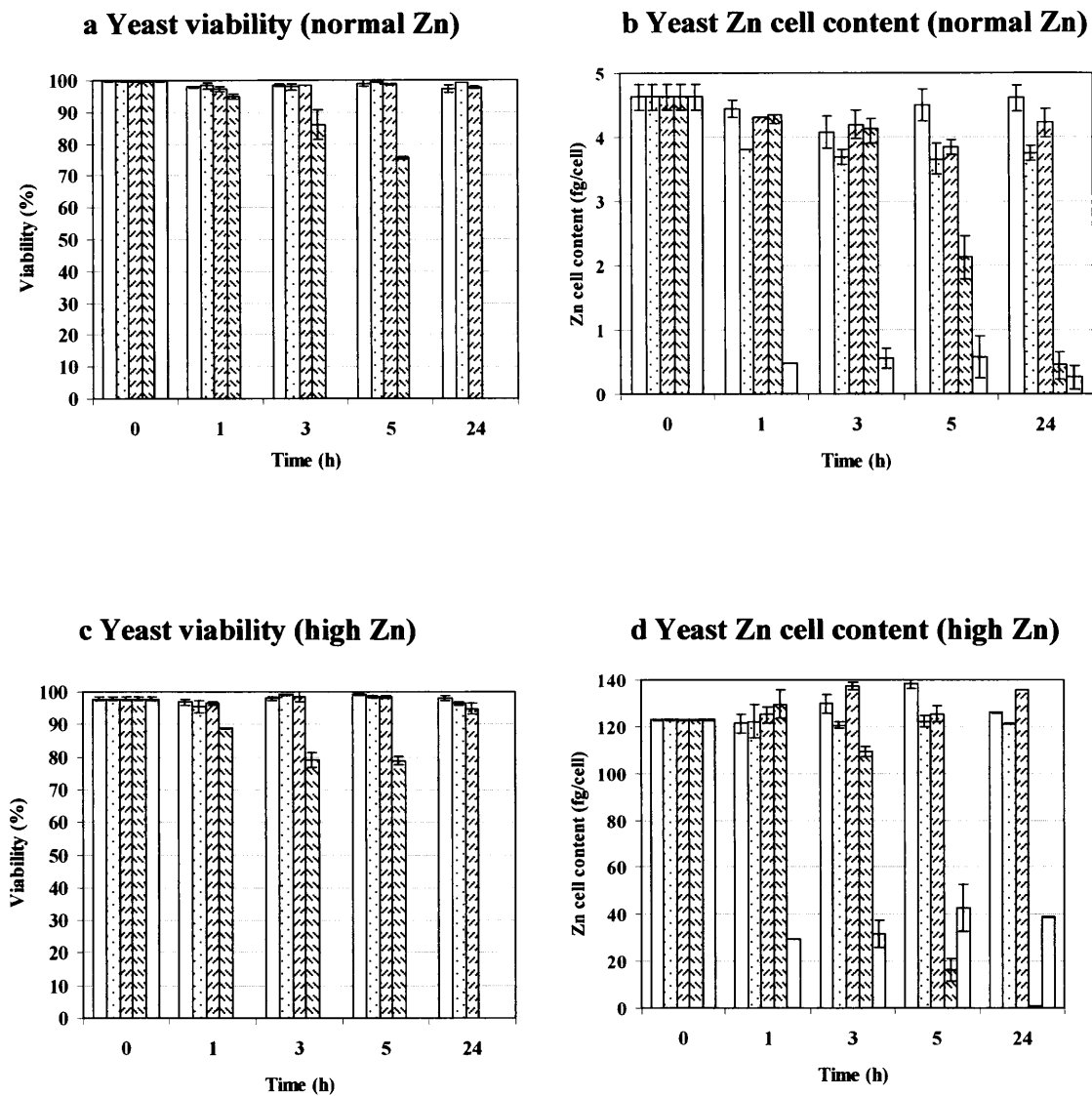
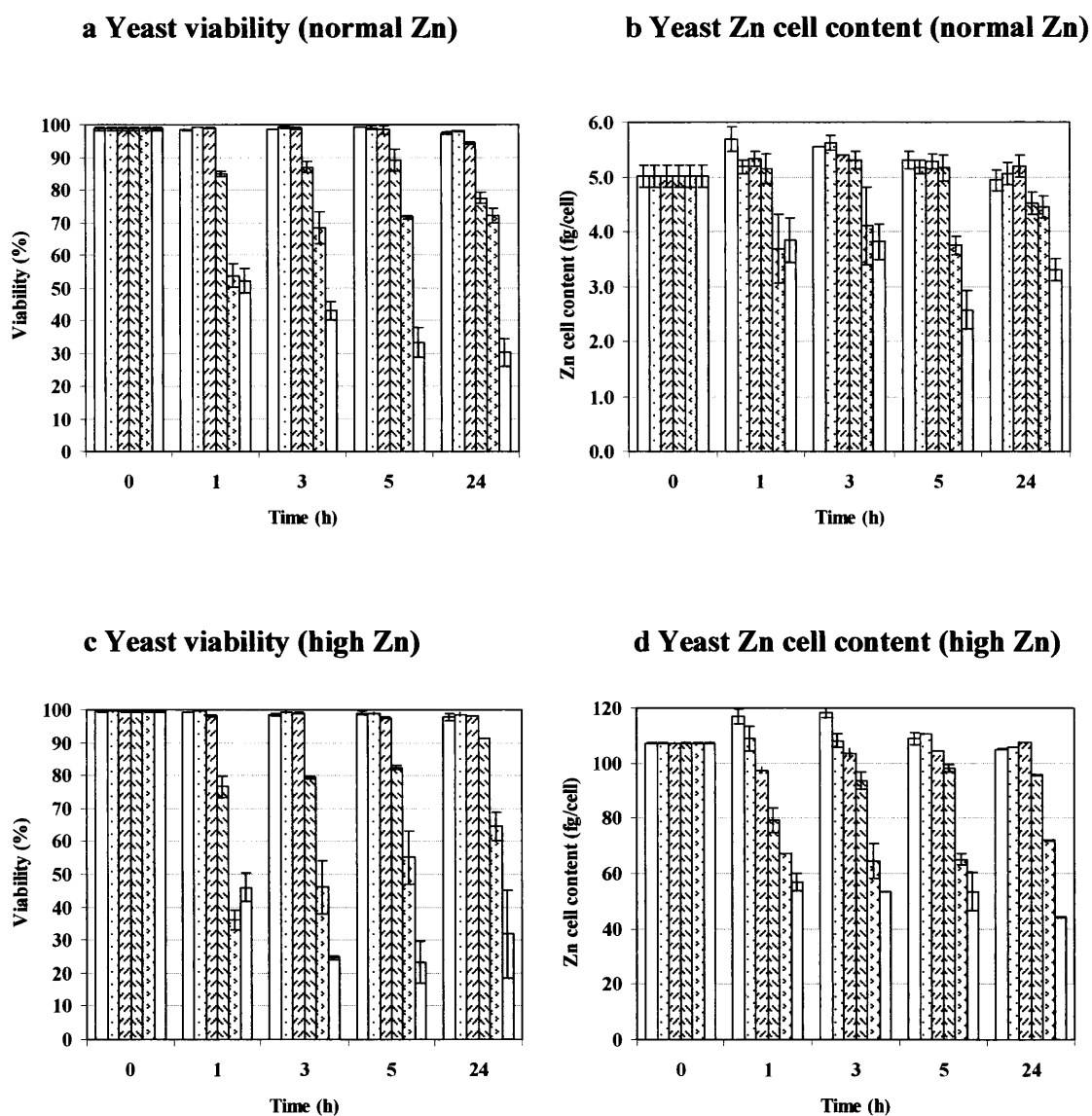


Figure 6.6 Zinc cell content and viability of brewing yeast under osmotic stress. Cells of the lager strain B were cultured in malt wort with 1.13 ppm (a, b) and 38.5 ppm zinc (c, d). Cells were harvested, washed once and resuspended in dH₂O at the following sorbitol concentrations: 0% (□), 2.5% (▤), 5% (▨), 10% (▩), 20% (▧) and 30% (▦) (w/v). Zinc cell contents (a, c) and viability (b, d) were checked in the first 5 hours and after 24 hours.



Zinc and water stress

During freeze-drying stress, all cells of the lager strain B died but they retained very high intracellular zinc content, about 75% of their original content (Fig. 6.7). The experiment was conducted without supplementation of any cryo-protectants. This was necessary to avoid any alteration of the zinc cellular levels and to investigate a possible protective role by zinc ions. The process employed in this experiment may be very similar to the process employed by Lallemand Inc (J. McLaren, personal communication) for the production of the commercial zinc supplement *Servomyces* (for details see paragraph 1.3.3). The level of zinc in this product was found to be 57,000 ppm compared with 1,279 ppm of the freeze-dried sample prepared with cells preconditioned in high zinc. It is conceivable that some differences in processes are performed to maximise zinc cellular concentration (e.g. growth medium may not be removed prior to freeze-drying) as well as the use of a yeast strain able to accumulate higher levels of zinc. The effect of the freeze-drying stress on yeast cells was probably similar to the osmotic stress. During the lyophilisation process, frozen cells were subjected to vacuum/sublimation to allow the intra-cellular water to pass from the solid to the gaseous state, skipping the liquid state. Yeast cells had to face a rapid freeze shock, followed by dehydration. The consequences of dehydration to the plasma membranes, previously discussed, are usually irreversible with cells producing reactive oxygen species to survive against this stress. Sometimes these compounds can cause damage to DNA and lipid peroxidation of membrane components (Espindola *et al.*, 2003). As a consequence, the viability decreased considerably. Unexpectedly, zinc content was not dramatically affected. The reason for such a phenomenon may be complex. It is possible that the quick freezing before dehydration caused stability in cellular compartments, including plasma membrane. During dehydration these compartments remained intact retaining most of the zinc. Other biological mechanisms such as oxidative stresses may have determined the high mortality considering that cells had to face the stress without the necessary time to metabolise stress protective molecules, such as glycerol or trehalose.

Yeast cells subjected to liquid nitrogen cryopreservation for 14 days maintained a good viability: 38% in cells preconditioned in normal zinc (4.13 fg/cell) compared with 64% in cells preconditioned in high zinc (85 fg/cell). The former cells maintained their original zinc intracellular levels while the latter lost 20% of zinc. Analyses on the cell topography

by AFM (courtesy of Dr. E. Canetta) showed that most of the cells with normal levels of zinc kept intact cell wall morphology after stress, with only some of them collapsing (Figs. 6.8.a; 6.8.b). Cells with high levels of zinc had altered roughness of the cell wall prior to stress. Although the fermentation performance of these cells was not tested, previous experiments (see chapter 5) showed that cells with high intracellular zinc levels had excellent fermentation performance. After stress, the morphology did not change but no collapsed cells were found (Figs. 6.8.c; 6.8.d) and cells had enhanced protection against cryopreservation. During freezing and thawing, cells are subjected to mechanical injuries due to formation of ice water crystals within the cell. Cells usually respond with a variety of “cell rescue” proteins, whose functions is to repair the damage to the cell envelope and to the cellular compartments. Antifreeze proteins may also be synthesised (Walker and Van Dijck, 2006). In this experiment, cells were protected by the addition of glycerol 20% and intracellular zinc certainly protected against the stress. The protective role of zinc may concern the stability of enzymes involved in stress protection. For example Carpenter *et al.* (1986) demonstrated that addition of zinc ions to organic solutes resulted in enhanced capacity to protect purified phosphofructokinase during cryopreservation. Magnesium and other cations did not show the same effects.

Figure 6.7 Zinc cell content and viability of brewing yeast after freeze drying treatment and liquid nitrogen preservation. Cells of the lager strain B were cultured in malt wort with 1.06 ppm (a, b) and 34.85 ppm zinc (c, d). Cells were treated in liquid nitrogen or freeze-drier and stored for respectively 14 days and 24 hours prior to viability and zinc cell analyses. The histograms represent: control in dH₂O (□), free-drying after 1 day (▨), free-drying after 10 day (▩) and liquid nitrogen after 14 days (▧).

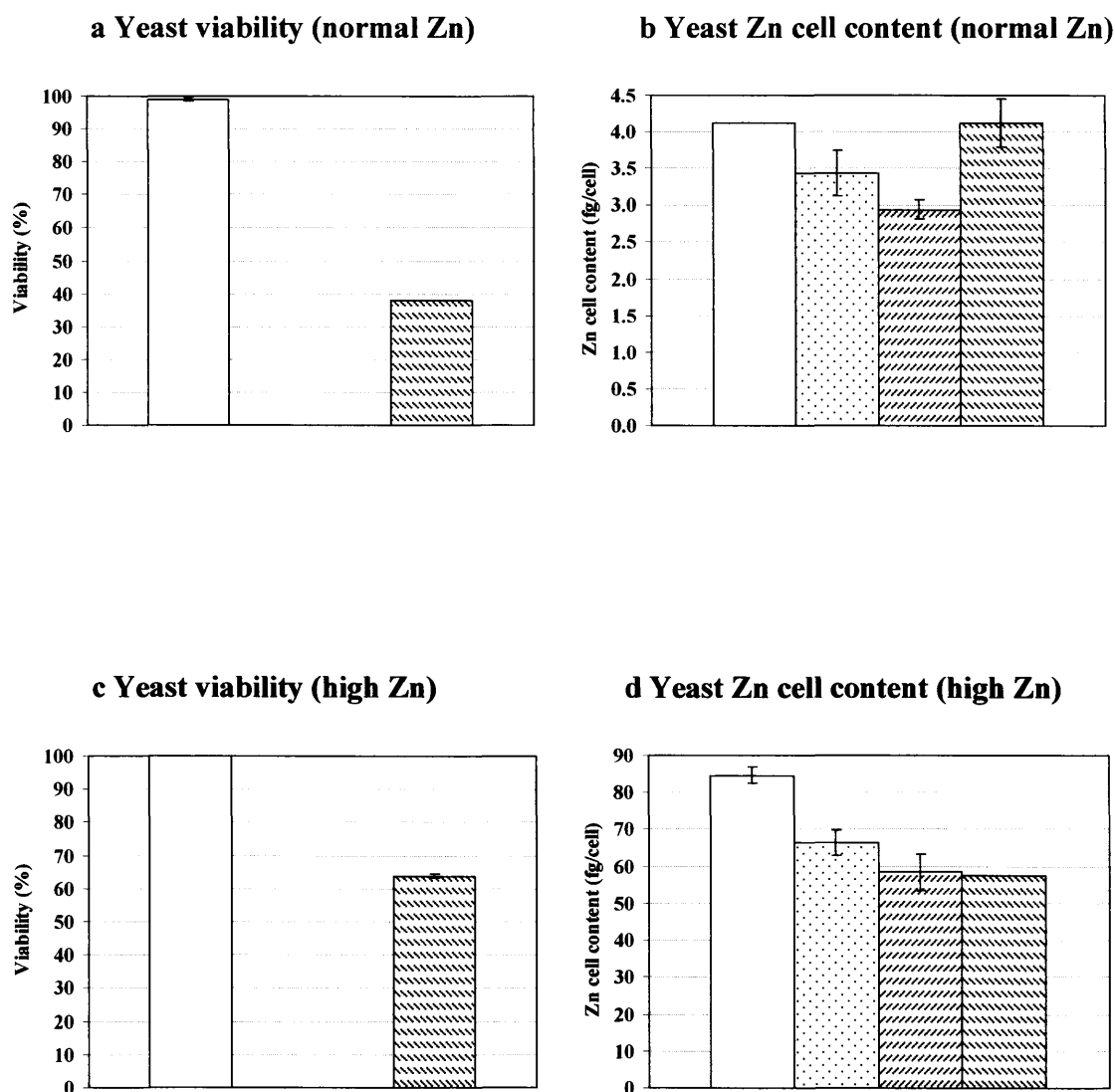
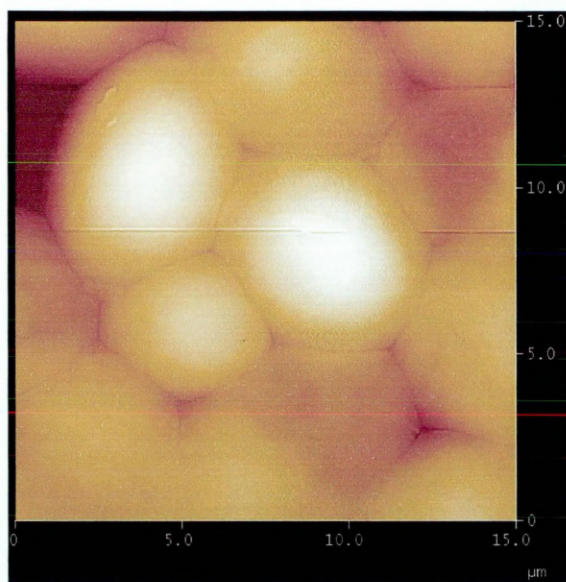
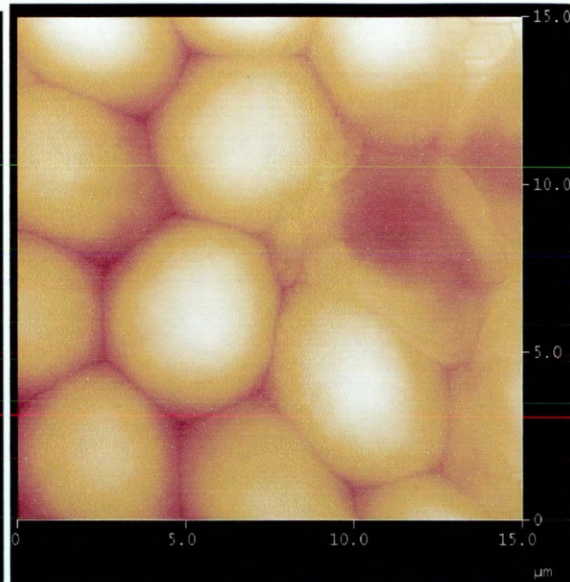


Figure 6.8 Atomic Force Microscopy of surface topography of brewing yeast cells after liquid nitrogen and freeze-drying stress. Yeast cells of the lager strain B with normal (4.13 fg/cell) and high levels of zinc (84.68 fg/cell) were treated in liquid nitrogen or freeze-drier and stored for respectively 14 days and 24 hours prior to microscopic analyses.

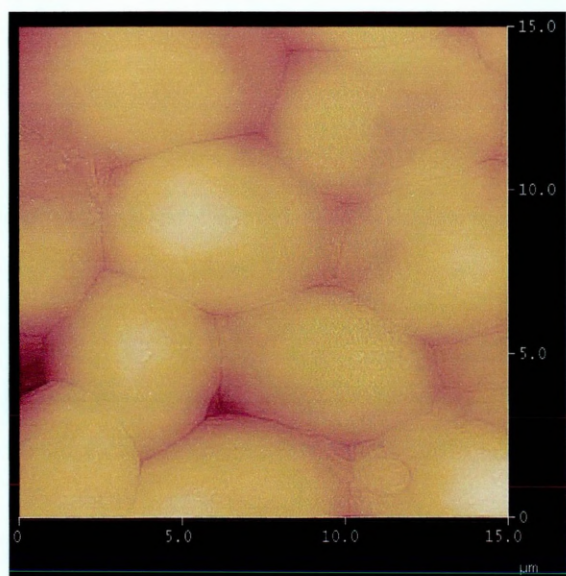
a Control cells (normal Zn)



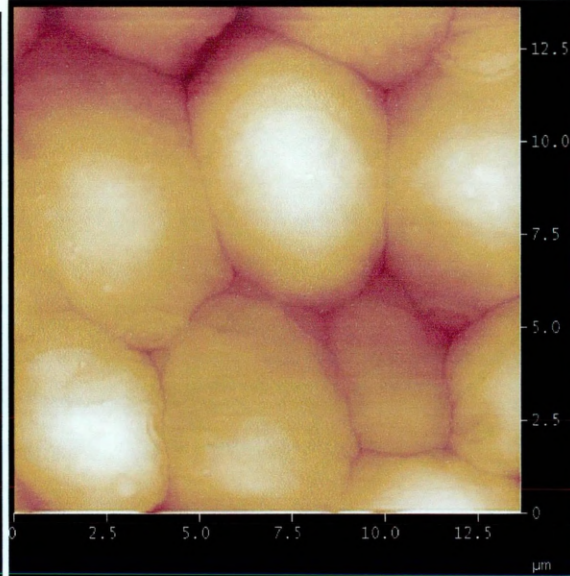
b Liquid nitrogen treated cells (normal Zn)



a Control cells (high Zn)



b Liquid nitrogen treated cells (high Zn)



Zinc and brewery- simulated stress

The small-scale yeast storage studies described how cells of the lager strain B responded to a combination of more stresses: high cell density, starvation, low temperature, CO₂ and ethanol. The use of commercial pasteurised beer during stress, guaranteed these combined parameters. Due to the technical difficulties to maintain a constant CO₂ pressure for a long period of time, the experiments were carried out for only 2 hours. In brewing, yeast cells could be stored in storage vessels for several days (depending on the brewery) prior to repitching. Cells with intracellular zinc levels of 3.38 fg/cell, did not change their viability but lost 12% of intracellular zinc (Fig. 6.9) when stressed (Zn from 3.38 to 2.82 fg/cell). It would be interesting to determine if such a release was only related to the shock due to the sudden exposure of cells to the new adverse environment or was just the beginning of a continuous and constant stress. Lentini *et al.* (2003) have examined the influence of such combined stresses on the yeast cell physiology during storage under high ethanol concentration (10%) and for 7 days. Intracellular levels of trehalose increased consistently, functioning as cellular protectant. Yeast vitality and viability decreased and the excreted protease levels increased reducing the levels of beer foam proteins. Moreover, the pH of the slurry increased and pH measurements were therefore considered a very good parameter to determine the cellular stress level during storage (Lentini *et al.*, 1998). This was probably due to the cellular uptake of H⁺, necessary to regulate the ion transport across the plasma membrane. Anyway in industrial scale, this phenomenon may be influenced by the high cellular density and the residual nutrients and energy available for the correct functioning of the ATPase. Prolonged exposure to CO₂ and therefore to anaerobiosis can also stress cells when lipids are not available in the medium. This can affect unsaturated fatty acids and ergosterol synthesis (Belviso *et al.*, 2004), thus influencing the subsequent fermentations.

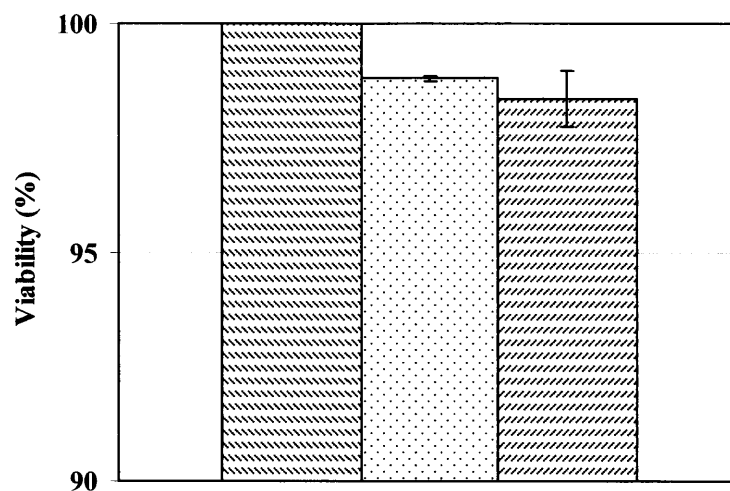
Experiments carried out under temperature, ethanol and combined temperature/ethanol stresses (Fig.6.10), showed the same kinetics described previously for viability loss and zinc release. The combination of these physical and chemical stresses had a synergistic effect on the viability of the cells which were all dead after only one hour from the beginning of the stress. Interestingly, magnesium and zinc cell contents were affected with similar patterns. The two metals were released during stress with some residual levels

remaining after 24 hours, probably due to tight bonds formed with the cell wall. The release of magnesium ions during such stresses was already observed in wine strains of *S. cerevisiae* by Birch (1997). In this study, magnesium ions were protecting yeast cells from the stresses and high viability was detected in cells preconditioned with magnesium or growing in medium with increased concentrations of magnesium. Probably magnesium acted as a membrane stabiliser (Birch and Walker, 2000; Hu *et al.*, 2003a). These observations were not found in yeast cells preconditioned with zinc during this Thesis (see Fig. 6.2 and 6.5), although a limited range of zinc levels was studied. Clearly, the release was not only unique to zinc ions but concerned also other divalent cations, suggesting that the phenomenon was more general. The decrease in ATP may be due both to a general alteration of the plasma membrane during stresses or to the mechanisms of protection activated by the yeast cells to face the environmental changes. Unfortunately, the bioluminescence method employed did not allow ADP measurements to confirm any of the two hypotheses. The results gained appeared to indicate that ethanol and temperature stresses influenced the plasma membrane fluidity allowing a general release of metal ions. In order to confirm this hypothesis, the use of the probe Laurdan (Learmonth and Gratton, 2002) was thought to give a good measurement of any change in cell membrane fluidity. Preliminary studies with this probe suggested that yeast membrane fluidity varies with culture age which may relate to metabolic status, increasing ethanol and decreasing sugar/nutrient levels. For details see chapter 5 “Studies on zinc influence on brewing fermentations, small scale conical vessels”.

The most severe stresses studied in this Thesis, at the longest exposure time, are depicted in Fig. 6.11. Loss of viability and zinc from cells of the lager strain B are shown in order to have a general overview of the effects of the stresses encountered in brewing. The techniques normally employed for the preparation of master cultures (freeze-drying and liquid nitrogen preservation) appeared to protect cells from the loss of zinc. Acid-washing and oxidative stresses had no effects on cellular viability and zinc cell content, while temperature (45°C for 24 hours), ethanol (20%v/v for 24 hours) and osmotic stresses (20%w/v for 24 hours) dramatically decreased both cell viability and zinc cell content.

Figure 6.9 Zinc cell content and viability of brewing yeast after a combined stress treatment of low temperature, high cell density and presence of CO₂. Yeast cells of the lager strain B were precultured in malt extract broth (Zn at 0.25 ppm), washed and responded in dH₂O (control) (□), in lager beer at high cell density, under CO₂, at 0°C and for 2 hours (▨) prior to analyses. Initial cell viability and Zn cell content are also represented (▩).

a Yeast viability



b Yeast Zn cell content

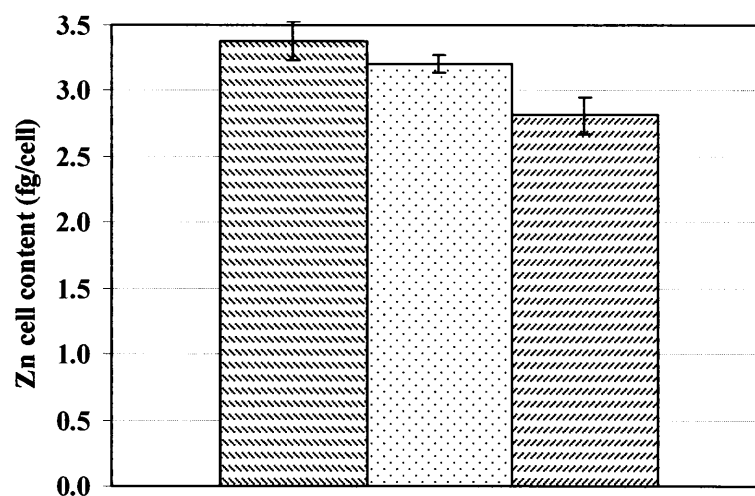


Figure 6.10 Viability, Zn, Mg and ATP cell content of brewing yeast after temperature, ethanol and combined temperature/ethanol stress. Yeast cells of the lager strain B were pre-cultured in malt wort (Zn at 0.46 ppm and Mg at 130 ppm) and resuspended in dH₂O at room temperature (□) (control), in dH₂O at 45°C (▤), in ethanol 20% (v/v) at room temperature (▨) and in ethanol 20% (v/v) at 45 °C (▩).

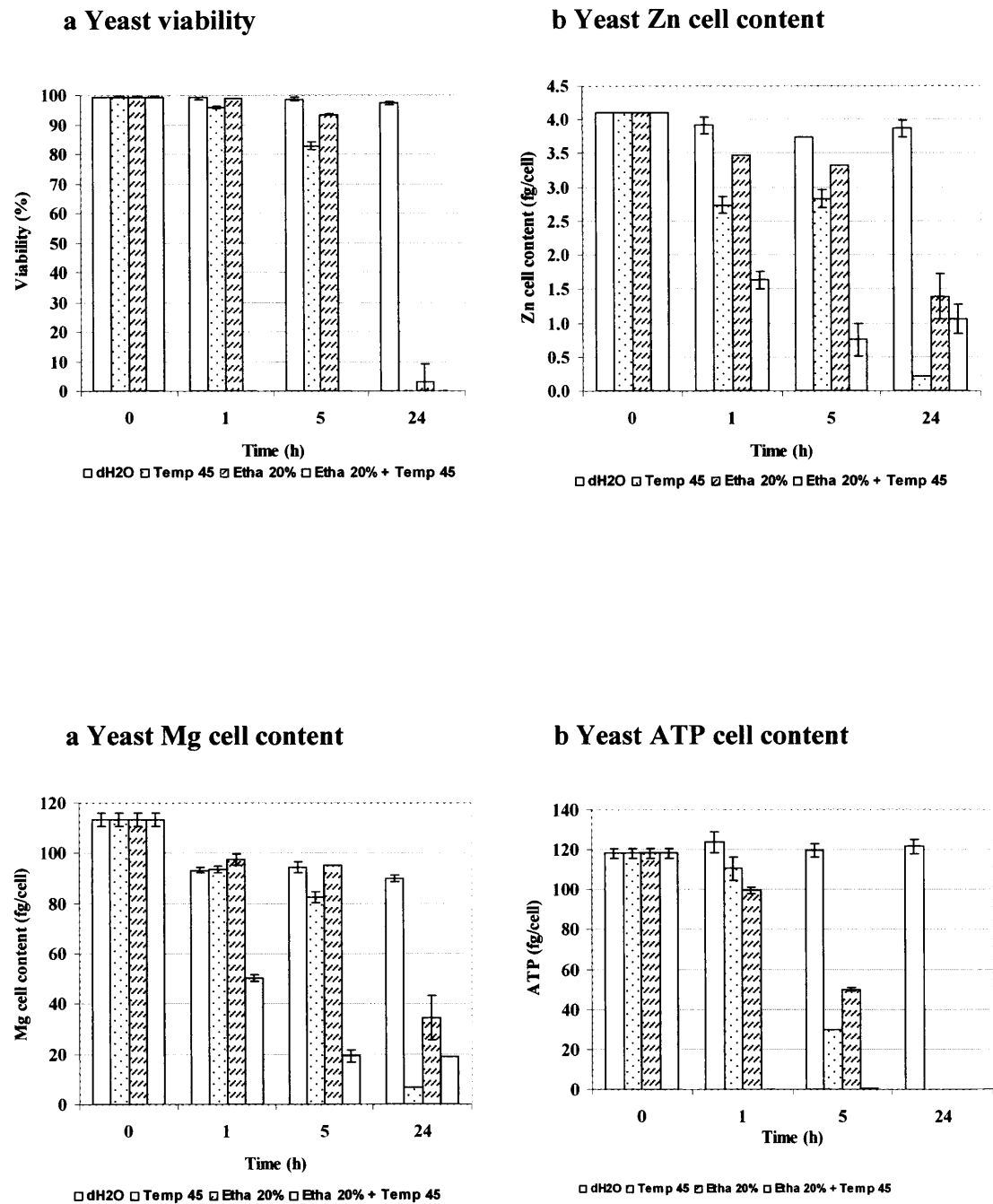
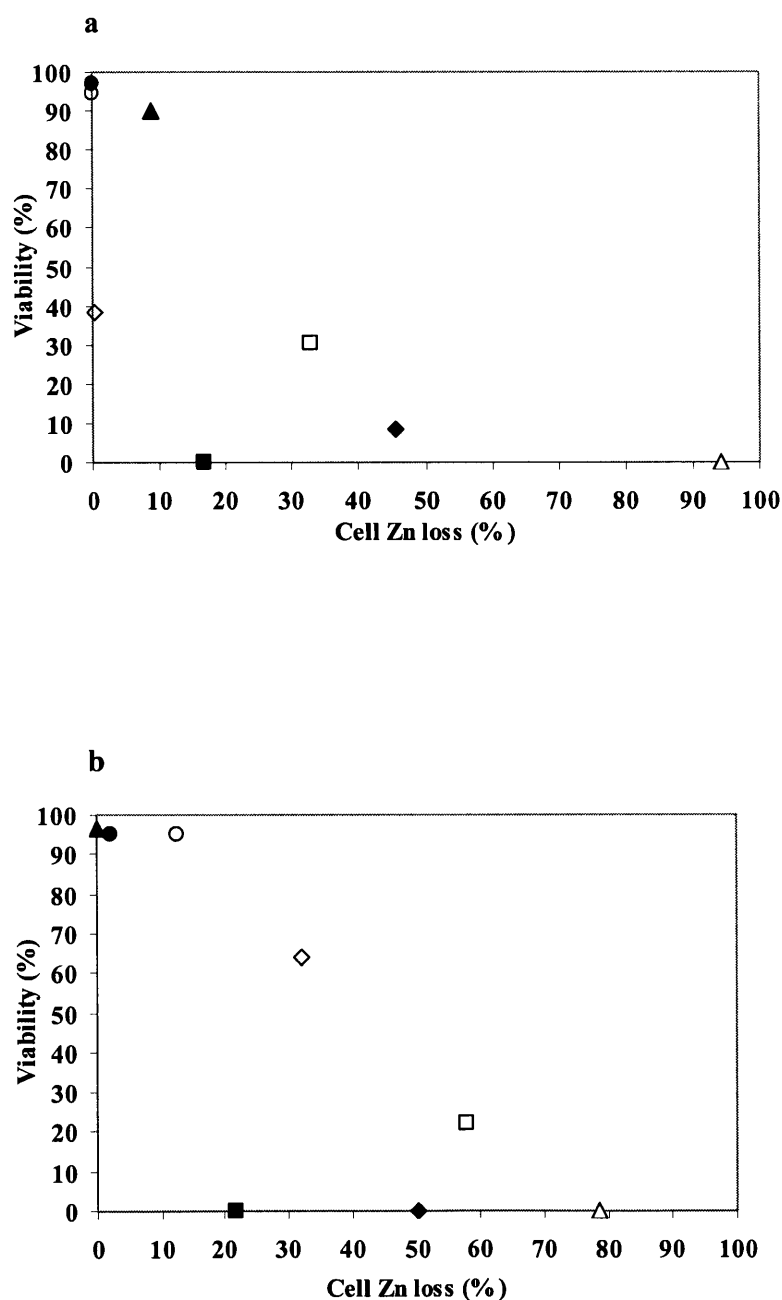


Figure 6.11 Comparison between cell viability and cellular Zn lost under various stress treatments in brewing yeast with initial normal and high Zn cell content. Yeast cells of the lager strain B were pre-cultured in normal (a) and high (b) Zn medium and stressed as described in this chapter. Figures represent the most severe stresses and at the longest exposure time. The symbols used represent the following stress conditions: ● persulfate wash pH 2.2-2 hours, ○ phosphoric acid wash pH 2.2-2 hours, ■ freeze-drying- 24 hours, □ sorbitol 30%-24 hours, ◆ ethanol 20%-24 hours, ◇ liquid nitrogen-14 days, ▲ peroxide 1%-24 hours, △ temperature 65°C-24 hours.



6.4 Summary

The results of the research presented in this chapter showed that:

- Yeast cells of the lager strain B insulted by oxidative and acid-washing stresses were not affected in terms of viability and intracellular zinc content, at the conditions herein studied.
- Severe and prolonged exposure to ethanol, temperature and osmotic stresses decreased both viability and intracellular zinc content of the lager strain B.
- Freeze-drying treatments, using the procedure described in this Thesis, killed the cells of the lager strain B but did not alter dramatically the intracellular zinc content.
- Cells of the lager strain B stored in liquid nitrogen maintained high viability, although they lose some of their intracellular zinc, if they were pre-conditioned in high malt wort zinc levels.
- Ethanol and heat stresses have a synergistic and accelerated effect on mortality and on released zinc cell content of the lager strain B.
- Yeast cells of the lager strain B killed by temperature, ethanol and temperature/ethanol stresses lose magnesium and reduced their intracellular ATP levels. This phenomenon might have depended on alterations in plasma membrane permeability.
- Generally, a relationship appeared to exist between cell zinc loss and decrease in viability.
- Control and correct management of the physico-chemical properties of the environment are essential to avoid stresses leading to diminished fermentation performance and zinc loss.
- Further investigations are necessary to establish the role of zinc in stress protection, including the determination of the optimal intracellular zinc content for plasma membrane stability during chemical and physical stresses.

Chapter 7

Studies on zinc limited growth of *Saccharomyces cerevisiae*

7.1 Introduction

Cultivation of yeast cells may be carried out in three modes: batch, fed-batch and continuous culture. In industrial applications aimed at producing specific metabolites, e.g. alcohol or alcoholic beverages, such as bioethanol, wine, beer and whisky, cells are generally cultivated in **batch culture**. This system is characterised by the growth of yeast cells in the medium until the nutrients have been completely consumed. Growth normally occurs in three phases: lag, exponential, and stationary phase. In batch phase, cells are exposed to continuous changes in environmental parameters including: dissolved oxygen concentration, nutrient availability, catabolite accumulation and pH changes. In industrial applications where biomass is the main product, such as baker's yeast production, fermentation (or, strictly speaking, propagation) is normally carried out in **fed-batch** mode. In this method, nutrients are delivered in a controlled manner in order to keep cells in exponential phase for a longer period, to avoid the Crabtree effect (see chapter 1.2.4) and to reduce the accumulation of catabolites (primarily ethanol) that may be toxic. Although at end of production, oxygen transfer may become limiting and the efficiency of growth is usually impaired due to the shift from respiratory to fermentative metabolism, fed-batch cultivations allow higher biomass production compared to batch cultures. The third system used for yeast cell cultivation is the **continuous culture**, where fresh medium is continuously added to the culture and simultaneously culture (cells plus growth media) excess volume is removed in order to keep the culture volume constant. This method prevents accumulation of toxic metabolites and ensures that the environment is kept constant. This particular condition, defined as steady state, is encountered when there is no change in biomass and in substrate composition.

Although studies in batch culture are close simulations of industrial processes aimed at producing alcohol, in research, continuous culture offers the invaluable advantage to

investigate the physiological and/or genetic responses of the culture by altering a single parameter, such as a particular nutrient (zinc in the present study).

Most of the studies on zinc deficiency have been performed in batch cultures, in shake flasks, and were aimed to investigate the physiological yeast response to zinc depletion in terms of growth, cellular protein (Obata *et al.*, 1996) and phospholipid synthesis (Iwanyshyn *et al.*, 2004). Fermentative performance and flavour profiles of zinc depleted cultivations have also been described in simulated industrial (brewing) environments where zinc concentrations were altered from very low levels, as in the present study, or in other authors' investigations (Helin and Slaughter 1977b; Raspor *et al.*, 1990; Mochaba *et al.*, 1996; Bromberg, 1997; Skanks, *et al.*, 1997; Rees and Stewart 1998; Villa *et al.*, 1999). Batch cultures were also employed in genetic studies whose aim was to determine the genes involved in zinc uptake at low zinc concentrations (Zhao and Eide, 1996a; Gitan *et al.*, 1998; Zhao *et al.*, 1998; Lyons *et al.*, 2000; Higgins *et al.*, 2003).

In conditions of zinc depletion, Lyons *et al.* (2000) have determined 46 changing (differentially expressed) genes, with half of them altered in the mutant *zap1Δ*, concluding that zinc deficiency pervasively affects yeast physiology. Studies conducted on a brewing yeast strain grown in conditions of zinc depletion (Higgins *et al.*, 2003), have shown that 76 genes were induced or repressed fivefold, including the *ZRT* genes (see also paragraph 1.5.2, chapter 1) and some genes encoding heat shock proteins. Two of these genes, *YOR387c* and *YGL258w*, have been found to be highly specific to zinc deficiency, unaffected by iron depletion, not induced by oxidative or carbon starvation and not expressed in *zap1Δ* mutants. The capability to be induced 2 hours after zinc starvation has made these genes possible valuable molecular markers to detect zinc deficiency in advance. Unfortunately, the interpretation of transcriptome profiles obtained from these experiments is very difficult due to the continuously changing environment of the batch cultures. An inter-laboratory study aimed at comparing the reproducibility of transcriptome analysis in chemostat cultures with that in shake-flasks, has demonstrated that the former has enhanced reproducibility. Since in transcriptome analyses experimental replication is fundamental, chemostat culture is essential to ensure strict control of growth conditions and an acceptable level of reproducibility (Piper *et al.*, 2002). Therefore, additional or different target genes may play important roles in zinc homeostasis under condition of zinc

depletion. Growth rate affects transcription and makes it difficult to compare various zinc availabilities.

Zinc limitation studies in chemostat culture have already been employed in physiological studies of the yeast *Candida utilis* (Lawford *et al.*, 1980) and in *Aureobasidium pullulans* (Krogh *et al.*, 1998). These studies aimed to determine the response of growth and cellular biomass to altered levels of zinc starting from zinc limitation. For example, Lawford *et al.* (1980) have shown that at constant dilution rate $D=0.4\text{ h}^{-1}$, with zinc levels below $4.5\text{ }\mu\text{M}$, yeast biomass decreased and residual levels of sucrose (as carbohydrate source) remained consistent in the medium. At this level of zinc in medium, cell associated zinc was $0.8\text{ nmol/mg dry weight/cells}$.

Transcriptome studies in chemostat cultures have been performed only with *Escherichia coli* with toxic zinc levels (Lee *et al.*, 2005). To date, no zinc limitation study on *S. cerevisiae* cultures has been conducted in chemostat to determine neither the physiological nor the transcriptome responses of this yeast to zinc deficiency.

7.2 Experimental

Experimental aims

The aim of this study was to determine the effect of zinc deficiency on yeast cell physiology in batch cultures and of zinc limitation on yeast cell physiology in chemostat continuous cultures.

The first model provided useful information on growth related to industrial conditions where cells are exposed to environmental and nutritional parameter changes.

The second model aimed to determine the culture conditions for more comprehensive studies on genes up- and down-regulated during zinc limitation. The determination of a biomarker, to be employed in industry and capable of revealing the status of zinc depleted cells, was the ultimate purpose of this study.

Experimental approach

The brewing yeast strain lager A was employed in batch studies in shake flasks.

The strain used in the continuous chemostat culture studies was the haploid wild type strain *S. cerevisiae* CEN.PK 113-7D, for which there is available an extensive database of transcriptome profiles, from chemostat culture studies in conditions of altered oxygen

availability (Linde *et al.*, 1999; Tai *et al.*, 2005) macronutrient limitation (Boer *et al.*, 2003; Tai *et al.*, 2005), CO₂ stress and pH effects.

Experimental procedure

Batch zinc-depleted culture (full details in Materials and methods section).

Seed culture (lager yeast B) in EMM3 wort synthetic medium (no Zn added)
for 48 hours and repitched 2 times in order to deplete cells from Zn



Inoculum of 2×10^6 cells/ml in EMM4 “biochelated” medium
in order to decrease further the cellular zinc level for 48 hours at 25°C



Inoculum in flasks containing Zn at 0, 0.01, 0.05, 0.10, 0.17 and 0.35 ppm
Growth for 57 hours



Sampling for cell count, viability,
Zn cell content, Zn supernatant and ethanol

Continuous zinc-limited culture

Seed culture (haploid yeast) in shake flasks for 24 hours and repitched
4 times in order to deplete cells from Zn



Inoculation of chemostat



Batch phase of ~ 18 hours



Start of continuous culture at pre-defined flow rate (0.1 h^{-1})



Pre-steady state of 4 volume changes



Pre-steady state of further 4.8 volume changes



Sampling for dry weight, off gas, HPLC analyses
(glucose, ethanol, pyruvate, glycerol, acetate, succinate) CO₂
RNA for microarray transcriptome profiles

7.3 Results and discussion

In initial shake flask experiments, zinc-depleted cells growing in zinc-free medium had the same growth rate as cells growing at higher concentrations. After 20 hours they reached the stationary phase while cells continued to grow up at higher concentrations. At the end of fermentation, their final cell number was half that found at more normal zinc concentrations (Fig.7.1). Unexpectedly, viability of cells growing in zinc free medium was eventually higher than at higher zinc concentrations where most of the budding cells were dead (Fig. 7.2).

Low zinc levels (Fig. 7.3) negatively influenced cell growth and shortened the exponential phase. In zinc depleted medium, Obata *et al.* (1996) have previously observed a decrease in protein content and yeast biomass together with absence of several soluble proteins, ascribing to zinc a key role in protein synthesis. Therefore, growth depression in zinc depleted medium may be due to impaired protein synthesis. Yeast cells starved of zinc may also uptake other metals. For example, Obata *et al.* (1996) showed an increasing calcium uptake resulting in cell flocculation. This phenomenon was not observed throughout the 55 hours of the present experiment. The affinity of zinc to other transition metals, such as manganese and iron, may explain that enzyme sites normally occupied by zinc may be replaced by other elements, resulting in impaired enzyme activity.

It is quite unlikely that the loss of viability depended on zinc shock because death was evident only at the end of fermentation. Most probably seed culture cells became adapted to a condition of zinc depletion and, although performed well in normal zinc levels, they faced a “zinc stress” resulting eventually in loss of viability. It may be possible that part of cell population lost the capacity to uptake zinc ions through the normal transport systems (Eide, 2003) and that excessive ion accumulation occurred. Zinc levels may have become toxic and lethal to yeast cells.

With regard to ion uptake, as expected, all zinc was taken up from the medium (Fig. 7.3) and accumulated into cells (Fig.7.4). Adaptation to zinc depletion did not compromise the capability of the remaining part of the yeast population to uptake this metal. Zinc content of cells growing in zinc-free medium had the same level at beginning of fermentation as after 33 or 57 hours, although cells divided and shared their intracellular zinc during cell division (as described in paragraph 4.5 “Intracellular zinc localisation by microscope visualisation”).

Since zinc contamination in media and flask glassware was unluckily, most probably very low experimental zinc levels were not detected by the AAS in use for metal analysis of this study.

Although in shake flask experiments part of the ethanol may be lost by evaporation, ethanol final yields (Fig. 7.4) gave a good indication of the influence of zinc on fermentative performance of yeast cultures. Ethanol produced in zinc free medium was half of the ethanol produced at the highest concentration (Zn at 0.35 ppm). This result was also supported by the previously described simulated brewing experiment where the Imhoff conical vessels ensured no ethanol loss throughout fermentation.

Results gained from the batch experiment showed that yeast cells growing in zinc depleted media may encounter problems both in growth and in fermentative performance. Although experiments on a bigger scale are required to confirm this finding, industries recycling yeast biomass for several fermentations (e.g. breweries and bioethanol industries) are warned to keep under regular control both intracellular and extra-cellular zinc levels and to implement zinc supplementation when needed. Zinc supplements should be added to the initial stages, such as in the propagation vessel, to avoid cells adapted to zinc depletion, facing a zinc shock resulting in loss of viability.

A more complete picture of the yeast physiology in response to zinc limitation was examined in continuous chemostat cultivation experiments in aerobic and anaerobic conditions. Interestingly, preliminary studies in batch culture using the haploid strain CEN.PK113-7D under conditions of zinc depletion showed that cells were found unable to grow when they were transferred from a medium at pH 4.7 to a medium at pH 6. The medium employed for these studies (see Tab. 2.3 Composition of minimal medium for chemostat continuous cultures) was not supplemented with zinc but most likely contained trace amounts of zinc contaminants from other ingredients. At pH 6, these trace amounts of zinc present in the medium, were probably not bioavailable to the yeast cells to sustain the growth because of binding to other medium components (e.g. phosphates; Lee *et al.*, 2005).

Figure 7.1 Growth of a brewing yeast strain cultured in “biochelated” synthetic wort medium (EMM4) at various critical zinc concentrations. The yeast lager B was made zinc depleted (as described in experimental procedure, paragraph 7.4), repitched in EMM4 at variable critical zinc levels (from 0 to 0.35 ppm of Zn) and grown for 57 hours.

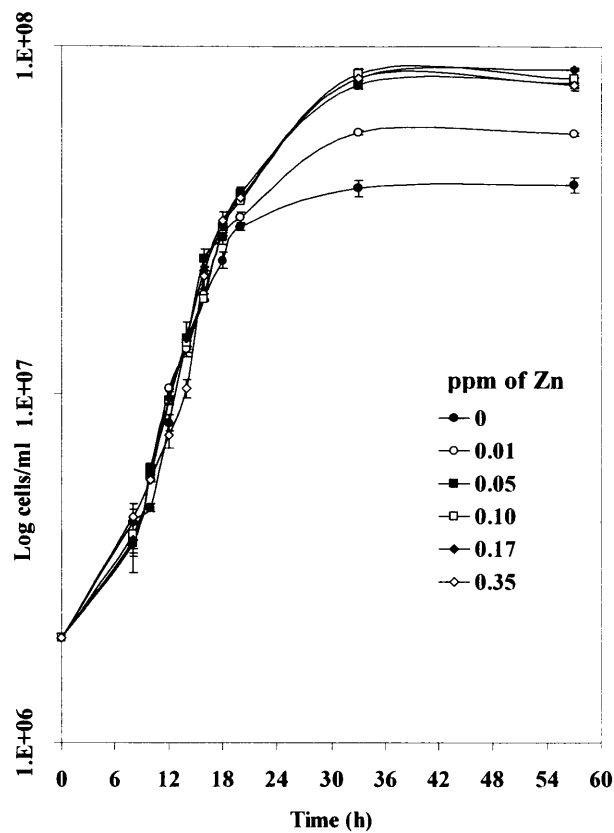


Figure 7.2 Viability of brewing yeast strain grown in “biochelated” synthetic wort medium (EMM4) at various critical zinc concentrations.

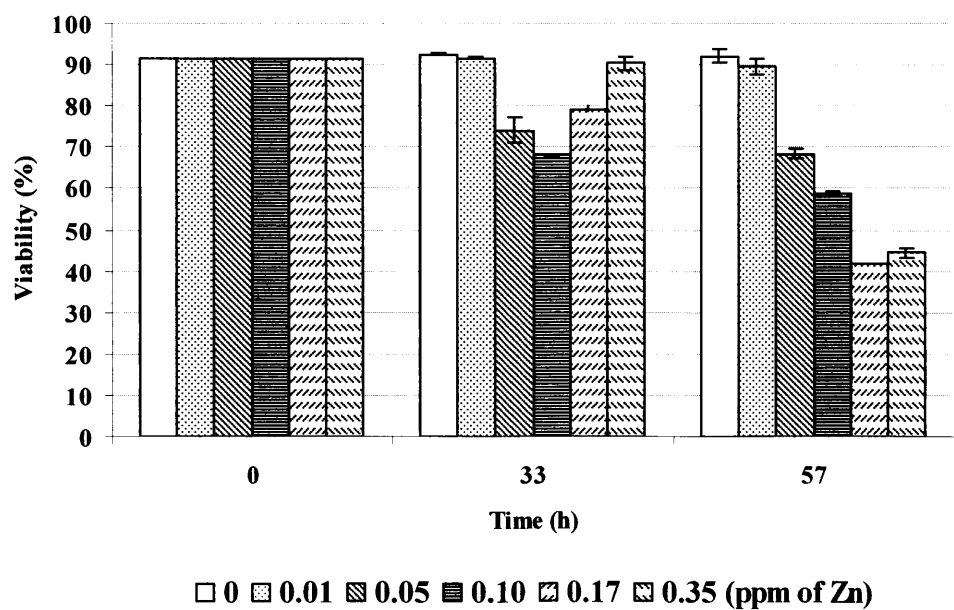


Figure 7.3 Zinc supernatant concentrations and zinc cell contents of a brewing yeast strain grown in “biochelated” synthetic wort medium (EMM4) at various critical zinc concentrations. Zinc levels were analysed by AAS in both the supernatant (a) and cells (b), throughout fermentation.

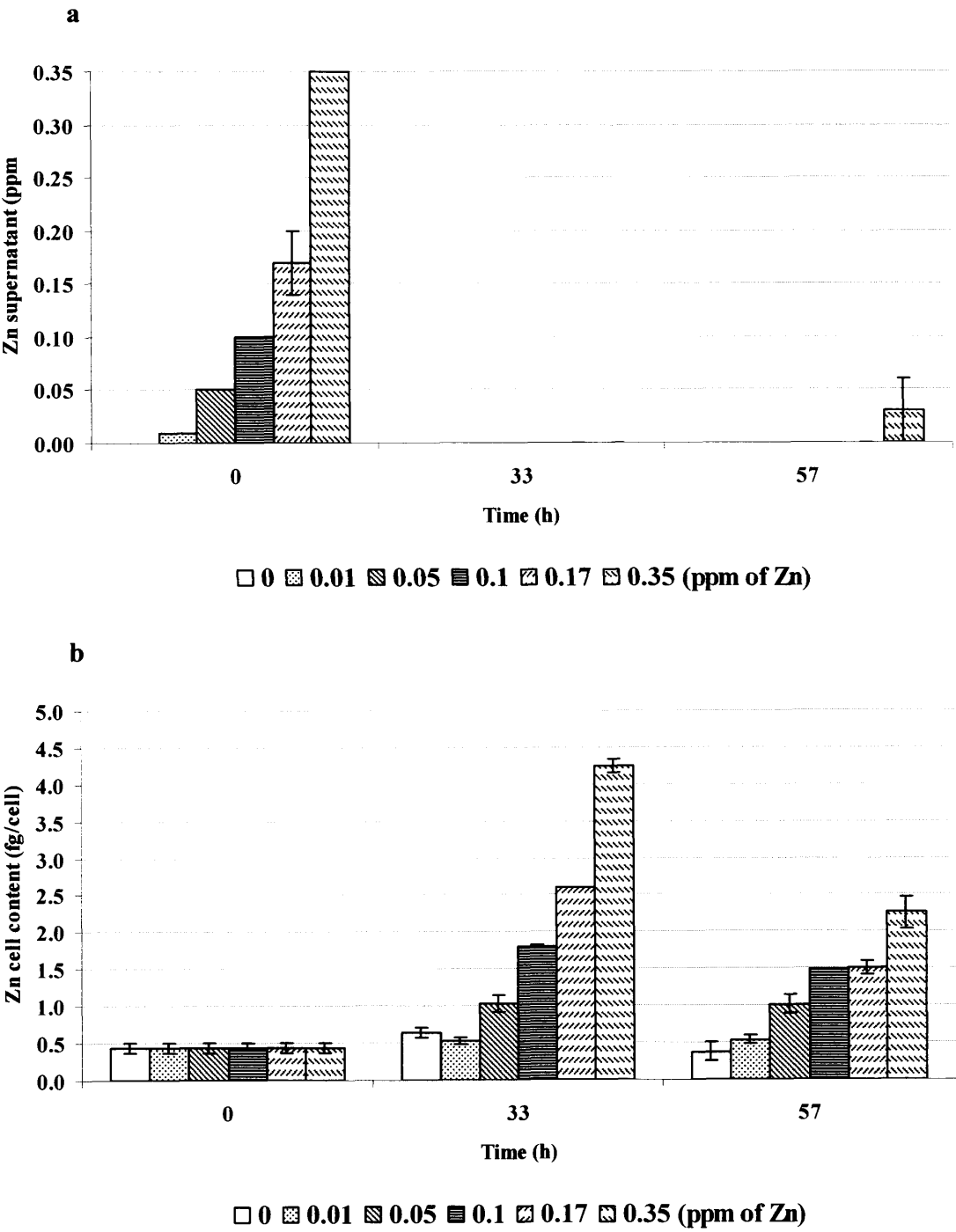
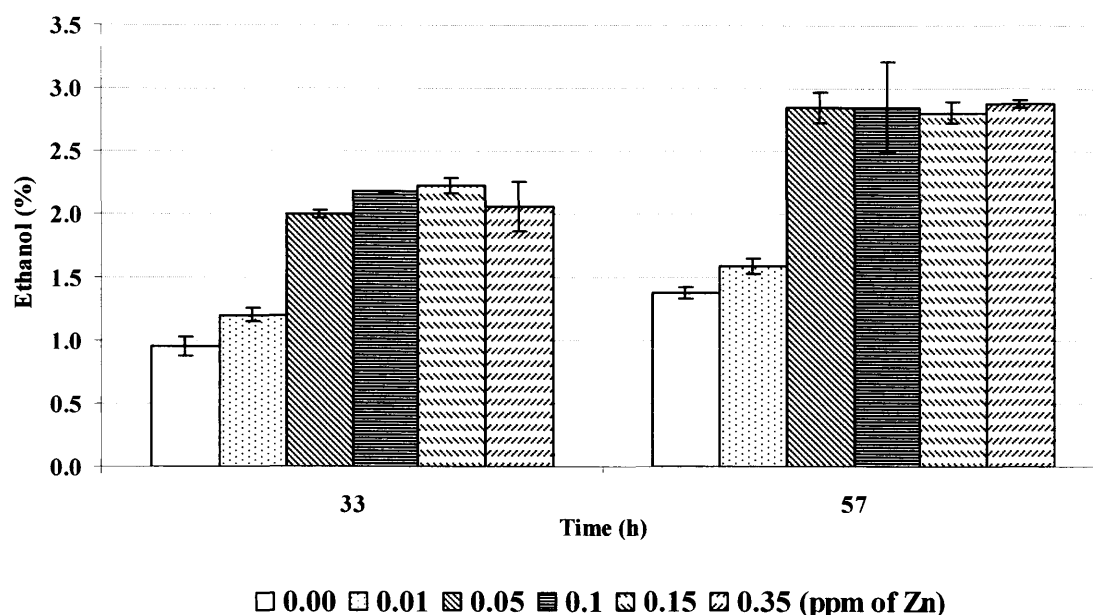


Figure 7.4 Ethanol levels during fermentation of brewing strain grown in “biochelated” synthetic wort medium (EMM4) at various critical zinc concentrations.



In anaerobic chemostat cultures, glucose is utilised solely by the alcoholic fermentation pathway, the reason being the complete absence of oxygen. The condition of zinc limitation was clearly demonstrated comparing data obtained from this study with those obtained from other nutrient limitation studies, e.g. carbon and nitrogen. Physiological parameters reported in Table 7.1 clearly showed that fermentative profiles of zinc limited cultures were similar to those found in nitrogen or carbon limited cultures (courtesy of Prof. J. Pronk). Dry weights and yield of biomass (g of biomass/g of glucose consumed) were also comparable among these studies. Carbon-limitation had a significantly higher biomass yield. Contrary to Zn and N-limitation, C-limited cultures exhibited no glucose repression. In the presence of N- and zinc-limitation, two phenomena are combined: absence of oxygen and excess of glucose, resulting in a lower yield of biomass. The acetate and glycerol production rates were higher under zinc-limitation, which may have revealed a redox problem. Higher acetate production could also indicate a limited capacity of ADH for ethanol production.

Proof of zinc limitation, in both aerobic and anaerobic conditions, was also given after a spike of 4.5 ppm of zinc sulphate, corresponding to 1.02 ppm zinc, performed when cells

were in steady state, in order to re-establish normal zinc levels. For example, in anaerobic conditions, after such a zinc spike, residual glucose levels decreased dramatically in 22 hours, from 24.71 g/L to 1.62 g/L, while dry weight increased from 2.49 g/L to 4.36 g/L, thus 75% more than the level encountered in conditions of zinc limitation. During the same period, CO₂ production also increased immediately (data not shown). It may be concluded that even in zinc-limited chemostat continuous culture, zinc uptake was very rapid. Although described in shake flask experiments, this phenomenon has already been investigated by MacDiarmid (2003) who defined it as “zinc shock”. This study allowed the determination of the gene *ZRT3* encoding the vacuolar protein transporter Zrt3, involved in the rapid accumulation of zinc into the vacuole. In this Thesis, increase in dry weight and CO₂ production and simultaneous decrease of glucose levels, following zinc spikes, clearly confirmed the experimental findings of the zinc limited batch experiments. Zinc has a key role in cell growth and metabolism of yeast cells. Zinc-depleted cells showed weak growth (lower biomass yield) and impaired fermentative pathway (lower CO₂ production). The reason for the former may be again found in the depression of the protein synthesis and cell division arrested in G1 phase of the cell cycle (Walker, 1998). The functioning of fermentative pathway enzymes and particularly of alcohol dehydrogenase (Magonet *et al.*, 1992) may be compromised leading to incomplete fermentation and high residual sugars. The immediate response of the culture to higher zinc levels clearly demonstrated that this metal was taken up very quickly even in continuous culture conditions. Generally, yeast cells adapted to zinc depletion maintained a high capacity of zinc uptake. Although zinc requirements were far lower than that of carbon and nitrogen, this study on zinc limitation has emphasised that the importance of this trace metal is not secondary to other elements, usually required at much higher concentrations, as shown for nitrogen.

In this study, the set up of zinc limited conditions of aerobic and anaerobic chemostats has generated very useful information for more in depth studies aimed to determine at the transcriptome level the genes up- and down-regulated during zinc limitation or after zinc spikes, when zinc conditions suddenly change from depletion to repletion.

Preliminary microarray studies on RNA samples of steady state cells in zinc-limited aerobic cultures and conducted in collaboration with Technical University of Delft (courtesy of Ms. L. Hazelwood), have determined 228 up-regulated genes during zinc limitation compared

with nitrogen and carbon limitations. Most of these genes were related to the zinc transport (*ZRT1*, *ZRT2*, *FET4*, *ZRT3*). One of these genes was *ZAP1*, the zinc regulator transcription factor. The same genes have already been identified to take part to the transport and homeostasis of zinc (Zhao and Eide, 1996a, 1996b; Gitan *et al.*, 1998; MacDiarmid, *et al.* 2000, 2002; Waters and Eide, 2002; Ellis *et al.*, 2004) (see also paragraph 1.5.2, chapter 1). Two genes, *YOR387c* and *YGL258w*, were found to be over-expressed respectively 217 and 167 times compared with the carbon and nitrogen-limited chemostat continuous culture conditions. These genes have already been identified by Lyons *et al.* (2000) and Higgins *et al.* (2003) in zinc depletion studies. Their functions are not known but they look particularly promising to be used as zinc-responsive molecular biomarkers during brewing process. Two more genes, *MUC1* and *ADH4*, were found to be highly expressed in zinc-limitation and are known to be involved respectively in yeast flocculation (see also paragraph 5.1) and synthesis of fusel alcohols, respectively. Interestingly, in aerobic conditions, higher concentrations of zinc were required for growth (0.05 μM of zinc were added) and this was thought to be dependent on the high requirements of zinc for the metallo-enzyme superoxide dismutase (Cu,Zn-Sod) in order to scavenge the potential harmful superoxide anion ($\text{O}_2^{\cdot-}$) during the aerobic stress (see also paragraph 6.5 “Zinc and oxidative stress”). The genes encoding this enzyme were not over-expressed, thus confirming that zinc has merely a structural role in this enzyme (Parat *et al.*, 1997).

The use of the wild type haploid strain CEN.PK 113-7D for such studies was of prime importance. The use of this strain provided an invaluable advantage of having available an extensive transcriptome profile database, previously gained using genome-wide analyses by microarray technology. The identified zinc-signature transcripts obtained by DNA microarray analyses may be subsequently tested for validity in industrial strains of *S. cerevisiae*, since this technique is very powerful to detect genotypic similarity between different yeast strains (Daran-Lapujade *et al.*, 2003). The ultimate aim is to determine a set of genes to be used as molecular biomarkers to detect zinc cellular deficiency during industrial processes (e.g. brewing).

Table 7.1 Physiological parameters of zinc-limited anaerobic (a) and aerobic (b) chemostat cultures of *S. cerevisiae*. The physiological parameters of the present study were compared to data obtained from other nutrient limitation. Data represent the mean \pm S.D. of data from four independent steady-state chemostat cultivations.

a			
	Zinc limited	Carbon limited	Nitrogen limited
Reservoir glucose g/Lt	57.5 \pm 0.25	25.2 \pm 1.71	65.1 \pm 0.13
Residual glucose g/Lt	20.2 \pm 0.60	0	18.2 \pm 1.6
Dry weight g/Lt	2.55 \pm 0.15	2.38 \pm 0.04	3.14 \pm 0.13
Y _{sx} (g/g) ^a	0.07 \pm 0	0.10 \pm 0.01	0.07 \pm 0.001
q glucose ^b	8.4 \pm 0.14	5.6 \pm 0.29	8.1 \pm 0.04
q ethanol ^c	13.8 \pm 0.25	8.8 \pm 0.4	13.5 \pm 0.7
q pyruvate ^c	0.04 \pm 0.01	0.008 \pm 0	0.03 \pm 0.004
q glycerol ^c	1.09 \pm 0.01	0.810 \pm 0.056	0.76 \pm 0.04
q acetate ^c	0.16 \pm 0.02	0.008 \pm 0.008	0.06 \pm 0.05
q succinate ^c	0.03 \pm 0.01	0.03 \pm 0.006	0.03 \pm 0.01
q CO ₂ ^d	15.5 \pm 0.5	10.01 \pm 0.5	15 \pm 0.8
q O ₂ ^e	NA	NA	NA
Carbon recovery (%)	101 \pm 0.90	102 \pm 4	99.4 \pm 0.6
Fermentative capacity ^f	11.1 \pm 0.12	7.6 \pm 0.5	13.8 \pm 0.65

b			
	Zinc limited	Carbon limited	Nitrogen limited
Reservoir glucose g/Lt	59.8 \pm 1.38	7.5 \pm 0.15	57.1 \pm 1.50
Residual glucose g/Lt	18.4 \pm 0.94	0.014 \pm 0.00	13.8 \pm 0.35
Dry weight g/Lt	4.3 \pm 0.14	3.6 \pm 0.09	4.1 \pm 0.07
Y _{sx} (g/g) ^a	0.10 \pm 0	0.49 \pm 0.01	0.09 \pm 0
q glucose ^b	5.36 \pm 0.27	1.1 \pm 0	6.44 \pm 0.35
q ethanol ^c	8.13 \pm 0.21	0	7.94 \pm 0.26
q pyruvate ^c	0.12 \pm 0	0	0.12 \pm 0
q glycerol ^c	0.08 \pm 0.01	0	0.08 \pm 0.01
q acetate ^c	0.07 \pm 0.01	0	0.03 \pm 0.01
q succinate ^c	0.04 \pm 0.01	NA	0.14 \pm 0
q CO ₂ ^d	12.34 \pm 0.24	2.8 \pm 0.3	12.3 \pm 0.53
q O ₂ ^e	2.80 \pm 0.06	2.8 \pm 0.3	3.42 \pm 0.08
RQ ^g	4.47 \pm 0.14	1.0 \pm 0.00	3.73 \pm 0.13
Carbon recovery (%)	105 \pm 2	98 \pm 3	96 \pm 0.41
Fermentative capacity ^f	12.7 \pm 0.41	14.8 \pm 0.10	9.7 \pm 0.40

Notes: ^a Yield of biomass (g of biomass/g of glucose consumed), ^b mmol of glucose consumed/g of biomass/h, ^c mmol produced/g of biomass/h, ^d mmol of carbon dioxide produced/ g of biomass/h, ^e mmol of oxygen consumed/g of biomass/h, ^f mmol of ethanol produced/ g of biomass/h, ^g RQ, Respiratory quotient (q_{CO₂} / q_{O₂}), NA not available.

7.4 Summary

The results of the research presented in this chapter showed that:

- In batch culture, in conditions of zinc depletion, the growth of the brewing strain B was not arrested but considerably impaired as was ethanol production.
- In chemostat continuous culture, in conditions of zinc limitation, the fermentative profiles of zinc limited cultures of the haploid strain CEN.PK-113D were similar to those found in nitrogen or carbon limited cultures.
- In anaerobic chemostat continuous culture, acetate and glycerol production rates were higher compared with nitrogen and carbon-limitations. This was probably due to a redox problem. Higher production of acetate may indicate a limited capacity of ADH for ethanol production.
- In zinc-limited chemostat continuous culture, in both anaerobic and aerobic conditions, zinc supplementation to cells in steady state produced an immediate increase in biomass yield, glucose consumption and CO₂ production. These changes were presumably due to immediate uptake of zinc ions by yeast cells from the growth medium.
- The establishment of zinc-limitation conditions in chemostat continuous culture has allowed the identification of Zn-signature transcripts obtained by DNA microarrays. The determination of up and down regulated genes during zinc-limitation will generate important information for both fundamental research and practical applications in biotechnology. The former is aimed at understanding the role of zinc in yeast physiology. The latter is aimed at determining specific sets of genes (zinc-responsive molecular biomarkers), to be used to detect zinc cellular deficiency in yeast during industrial processes.

Chapter 8

Concluding discussion

The aim of this Thesis was to gain deeper understanding of the influence of zinc on cell physiology of the yeast *Saccharomyces cerevisiae*. A selection of industrial yeast strains and a brewing strain were investigated with regards to: zinc uptake, intracellular zinc localisation, fermentative performance at variable zinc levels, zinc homeostasis under various stresses and yeast physiological responses to conditions of zinc limitation. The experimental approaches employed in this Thesis included preliminary laboratory experiments with shake flasks aimed at defining yeast cellular physiological response under strict control of zinc levels. The use of 1 L conical vessels and 200 L brewing fermenters allowed verification and extension of the initial laboratory findings to industrial scale. Chemostat continuous culture employed in zinc-limitation studies was necessary to ensure the strict control of growth conditions and an acceptable level of reproducibility for more in depth studies related to transcriptome analysis.

This research has generated fundamental information related to the yeast cell physiology. Yeast cells were found to uptake zinc from the growth medium very quickly within the first hour in shake flask experiments and within few hours in 1L conical vessel experiments. Zinc uptake kinetics were not strain dependent and also not related to growth medium, type of zinc salts supplemented and presence of calcium. Inhibition of the protein synthesis did not affect zinc uptake. Nevertheless, zinc uptake kinetics were influenced by temperature and cellular status, with dead cells unable to uptake zinc. Zinc uptake was metabolism-dependent and most likely regulated by plasma membrane H^+ -ATPase activity (Jones and Gadd, 1990). A low percentage of zinc was found to be bound to the cell wall, which as a consequence had not a key role during zinc uptake. Instead, zinc ions were quickly translocated into the yeast cell and stored into the vacuole. This accumulation allowed the zinc cellular concentration to be shared from mother to daughter cells at cell division. However, this process effectively lowered the zinc cell content at the end of fermentation. In brewing simulated small scale experiments with the lager strain B, 0.4- 1 ppm zinc resulted in fastest fermentation rates. Being strain and environmental dependent (Rees and Stewart, 1998), this range is not intended to be proposed as optimal for all breweries. The

concentrations tested (up to 23 ppm) did not have an impact on cell viability in either brewing or wine yeasts studied. The latter released part of the zinc into the medium at the end of the fermentation, probably because the relatively high ethanol levels produced affected plasma membrane fluidity (Learmonth and Gratton, 2003). In this regard, in brewing fermentations, yeast membrane fluidity varied with culture age. A complex relationship was found to exist between yeast cell zinc status and membrane fluidity, with cells having low zinc levels exhibiting low GP levels and high membrane fluidities. In pilot plant experiments, yeast fermentation performance appeared satisfactory at the zinc concentrations (range from 0 to 10 ppm) tested. This was due to the high levels of intracellular zinc of the seed culture. Thus, zinc-preconditioned yeast cells were found to perform well even in zinc free malt wort. Moreover, high zinc levels (10 ppm) influenced ester and higher alcohol profiles in beer, indicating a positive influence on the yeast pathways leading to these compounds. While oxidative and acidic stresses, under conditions normally encountered during the brewing process, did not alter yeast viability and zinc homeostasis, severe and prolonged exposure to ethanol, temperature and osmotic stresses impacted both on viability and cellular capacity to retain zinc. This phenomenon was not unique to zinc since magnesium (and ATP) were also lost during these insults. It is concluded that changes in membrane permeability were the primary cause for such metal release. Yeast cells were killed by freeze-drying but this treatment did not alter intracellular zinc content probably because the quick sublimation of water resulted in intact and stable cellular compartments, including the plasma and tonoplast membranes. Higher concentrations of zinc appeared to be protective when cells were stored in liquid nitrogen, although the cell wall topography roughness of zinc-enriched cells changed even prior to the stress. The protective roles of zinc during cryopreservation may be related to the stability of the enzymes normally affected by this stress. Zinc deficiency negatively influenced growth of brewing yeast in batch cultures with cells in zinc free medium exhibiting just half of the growth compared with normal zinc concentrations (0.35 ppm). Zinc-limited chemostat continuous culture of the haploid yeast strain CEN.PK113-7D also highlighted that yeast cell growth was strongly dependent on available zinc. This culture showed several similarities compared with nitrogen or carbon limited cultures, but in anaerobic conditions, acetate and glycerol rates were higher, indicating a possible redox problem. It is conceivable that zinc limitation negatively affected ADH for ethanol production, since the production of acetate was high. Zinc uptake has been proved to be quick even

under conditions of zinc limitation with cells immediately increasing biomass, glucose utilisation and CO₂ production after a zinc spike. Conceivably, all the transporters were activated allowing a quick uptake of zinc ions. This phenomenon was referred to as “zinc shock”. The set up of zinc limitation conditions in chemostat continuous culture (for the first time) allowed more in depth studies on up- and down-regulated genes during zinc limitation. The discovery of these genes can pave the way to studies on new pathways where zinc plays key roles.

The findings of this research also provide important practical knowledge. Since active metabolism is required during zinc uptake, it is suggested, during the brewing process, to supplement zinc salts to the yeast cell in optimal metabolic status and when sugars are abundantly available as sources of available energy. This condition is usually encountered at pitching time. Brewers are warned to avoid zinc additions when the temperature is too low, cell density is too high and the nutrients are limiting; for example, during yeast slurry storage or acid-washing. Since no major differences were found in terms of zinc uptake using different zinc salts, it is therefore advisable to use the cheaper salt. The range 0.4-1 ppm zinc appeared to be optimal for fermentation performance of the lager strain B, although in pilot plant brewery condition it was clearly shown that zinc-preconditioned cells performed excellently even in zinc free-medium. Higher zinc concentrations slightly delay the fermentation rates but may increase the synthesis of some esters and higher alcohols, improving beer taste and aroma. Further studies are required to elucidate the role of zinc in these pathways. Breweries using different strains from the one employed in this study (lager B) would be advised to perform series of tests aimed at verifying the best zinc concentration for the brewing strain employed. During the fermentation process, at any stage, brewers should control the status of their yeast cells and avoid any undue stresses. These may lead to diminished fermentation performance and zinc loss. The practice to recycle yeast biomass for several fermentations (as well as in the bioethanol industry), should be carried out carefully. A fraction of the yeast cone is usually discarded and this may cause the loss of a part of the yeast biomass representing an important reservoir of zinc. A specific set of genes (zinc-responsive molecular biomarkers) over-expressed during zinc limitation in chemostat continuous culture, could be potentially used during the brewing process to detect zinc cellular deficiency. Although this test may be expensive, this technique may be valuable for trouble-shooting problems occurring during industrial fermentation.

Suggested lines of future work:

- Interaction of zinc with other metal ions such as Mg or Cu. During industrial processes, zinc may have a synergistic effect on plasma membrane stability together with Mg and may antagonise the toxicity produced by Cu.
- Investigations into the preconditioning of yeast cells with zinc. Simulation of propagation in small fermenters, to determine the best conditions for yeasts to rapidly accumulate intracellular zinc.
- Extension of flow cytometry studies to determine how zinc is distributed into the cell population at various stages of the fermentation.
- Studies on the influence of zinc supplementations on ester and high alcohol profiles in alcoholic beverages.
- Studies on zinc content of cells at various fractions of the yeast cone at the end of the fermentation, in industrial scale brewing fermenters (e.g. 20 Hl).
- Studies on zinc content of cells from the storage tank of a brewery, to determine how the combination of high cell density, nutrient starvation, high ethanol and low temperature can influence cell viability and zinc cellular homeostasis.
- Further investigations to establish the role of zinc in stress protection, including the determination of the optimal intracellular zinc content for plasma membrane stability during chemical and physical stresses.
- Investigations into the effects of the dehydration stress on zinc cellular homeostasis. This study would generate important information for companies producing dried yeast biomass for industrial applications (e.g. baking, brewing, winemaking).
- Extension of molecular genetic (transcriptome) analysis of brewing yeast cells grown under conditions of controlled zinc availability in chemostats. This study will require the use of a brewing strain whose genome has been completely sequenced (Weihenstephan Nr.34; Nakao *et al.*, 2003).

Chapter 9

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